DIFFERENTIATION OF *PREVOTELLA INTERMEDIA* FROM *P. NIGRESCENS*, INCLUDING ISOLATES FROM PAPILLON-LEFÈVRE SYNDROME

A thesis submitted to the University of Manchester for the degree of Ph.D. in the faculty of Science and Engineering.

School of Biological Sciences

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ABSTRACT OF THESIS submitted by Katherine Louise Robertson for the Degree of Ph.D. and entitled 'Differentiation of *Prevotella intermedia* from *P. nigrescens*, including isolates from Papillon-Lefèvre Syndrome', Month and Year of submission June, 1999.

The work contained within this thesis is divided into two topics; (i) molecular DNA techniques for the differentiation of *P. intermedia* and *P. nigrescens* and (ii) identification of bacterial isolates from the oral cavity of Papillon-Lefèvre Syndrome (PLS) patients. This included isolates identified as *P. intermedia* using Rapid ID 32 A which were subsequently differentiated into *P. intermedia* and *P. nigrescens* using DNA techniques, therefore linking the 2 topics together.

The putative periodontopathogens *P. intermedia* and *P. nigrescens* are phenotypically similar but genetically distinct (Shah and Gharbia, 1992). Commercial identification kits cannot differentiate them and techniques including DNA-DNA hybridisation are used. In the first part of this study the potential of RAPD-PCR (random amplification of polymorphic DNA by polymerase chain reaction), partial 16S rRNA gene sequencing and species-specific PCR primers as methods of differentiating between the closely related species was investigated. Characterised strains of each species as well as clinical isolates (from PLS) identified as *P. intermedia* were examined. RAPD-PCR with primer L10 amplified marker bands which allowed the differentiation of *P. intermedia* from *P. nigrescens*, amplified bands were scored as present or absent and hierarchical cluster analysis resulted in two distinct species-specific clusters. The amplification of marker bands allowed successful typing of clinical isolates confirmed by cluster analysis. Partial 16S rDNA sequencing allowed the most likely identification of an isolate to be determined in relation to known strains; the results for clinical isolates correlated with RAPD-PCR data. Species-specific oligonucleotides were designed based on 16S rDNA sequence information. They were used as one of a primer pair with a universal primer (Choi *et al.*,1994) and tested against strains of each species in parallel with published oligonucleotides, 1Bi-1 and 2Bi-1(Dix *et al.*, 1990). Experiments were preliminary but the primers yielded inconsistent or non-specific amplification, making them unsuitable for species identification without further investigation. The similarity of PINLOs (*P. intermedia* and *P. nigrescens* like organisms) to *P. intermedia* and *P. nigrescens* was assessed using the same techniques.

PLS is a rare disease characterised by thickening of the skin on the palms of the hands and the soles of the feet and precocious periodontal destruction. The aim of this section of work was the identification of microorganisms isolated from oral sites of two PLS patients. Pure cultures of bacterial isolates were identified using commercial identification kits, traditional identification tests and Gas-Liquid Chromatography of fermentation products. Unidentified isolates were subjected to partial 16S rDNA sequence analysis and a FASTA search was used to determine the most likely identification. Aerobically growing organisms identified consisted mainly of *Neisseria* species and obligate anaerobes included *Peptostreptococcus* species and *Prevotella* species. The majority of isolates were facultative anaerobes and microaerophiles including *Actinomyces*, *Gemella* and *Streptococcus* species. Spirochaetes were observed by microscopy.

It was concluded that (i) RAPD-PCR and partial 16S rRNA gene sequencing allowed the differentiation of *P. intermedia* from *P. nigrescens*. (ii) The microbiology of PLS resembles that of periodontitis but that recognised periodontal pathogens e.g. *A. actinomycetemcomitans* may not be necessary for the disease to progress. (iii) Clinical isolates identified as *P. intermedia*, can be differentiated into *P. intermedia* and *P. nigrescens* by RAPD-PCR and partial 16S rDNA sequencing, which confirmed the utility of the techniques for species differentiation and an association of both *P. intermedia* and *P. nigrescens* with PLS.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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ABBREVIATIONS

A	Adenine (a purine)
A	Clinical (PLS) isolate A; **1** *2B*; *P. nigrescens*
ANUG	Acute Necrotizing Ulcerative Gingivitis
API	Appareils et procedes d'identification
AP-PCR	Arbitrarily primed polymerase chain reaction
ATCC	American type culture collection
ATP	Adenosine triphosphate
AWDMCS	Acid-washed dimethylchlorosilane treated (chromosorb for GLC)
B	Clinical (PLS) isolate B; **2** *3A*; *P. intermedia*
BHI	Brain heart infusion (broth)
bp	Base pairs
C	Cytosine (a pyrimidine)
C	Clinical (PLS) isolate C; **3** *2A*; *P. intermedia*
CBA	Columbia blood agar
D	Clinical (PLS) isolate D; **5** *1D*; *P. nigrescens*
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddNTP	Dideoxynucleotide triphosphates
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
Eh	Measure of redox potential
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPS	Extracellular polysaccharide
EtBr	Ethidium bromide
FAA	Fastidious Anaerobe Agar
FAB	Fastidious Anaerobe Broth
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced FAD an electron carrier in redox reactions

FID	Flame ionisation detector
FUM	Long term storage modified fluid medium (Loesche *et al.*, 1972; Gmür and Guggenheim, 1983)
G	Guanine (a purine)
GCF	Gingival crevicular fluid
GJP	Generalised juvenile periodontitis
GLC	Gas-Liquid Chromatography
h	Hour (s)
ID	Identification
IgA	Immunoglobulin (antibody) class A
IgG	Immunoglobulin (antibody) class G
IgM	Immunoglobulin (antibody) class M
LJP	Localised juvenile periodontitis
L10	Arbitrary primer
MAbs	Monoclonal antibodies
$MgCl_2$	Magnesium chloride
Mg^{2+}	Magnesium ions
min	Minute
MLEE	Multilocus enzyme electrophoresis
MRI	Manchester Royal Infirmary
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD an electron carrier in redox reactions
NCTC	National collection of type cultures
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PINLO	*P. intermedia* and *P. nigrescens* like organisms
P-int	*P. intermedia* PCR primer
PLS	Papillon-Lefèvre Syndrome
PMNs	Polymorphonuclear leukocytes
P-nig	*P. nigrescens* PCR primer
RAPD-PCR	Random amplification of polymorphic DNA by PCR

rDNA	Ribosomal DNA, copy of rRNA produced by PCR
REA	Restriction endonuclease analysis
RE- RTU3	General bacterial primer (Choi *et al.*, 1994)
RE-TPU1	General bacterial primer (Choi *et al.*, 1994)
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RSP	Reverse sequencing primer
16S RNA	RNA found within small subunit of prokaryotic ribosome
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp.	Species, (one of)
spp.	Species, (plural)
STE	Sucrose-tris-EDTA
T	Thymine (a pyrimidine)
Taq	DNA polymerase from the bacterium *Thermus aquaticus*
TCD	Thermal conductivity detector
TE	Tris-EDTA
T_m	Temperature
TPE	Tris-phosphate-EDTA
Tris	Trishydroxymethylaminomethane
Tris-Cl	Tris-chloride
TYC	Tryptone Yeast Cysteine Agar
UPMGA	unweighted pair-group method using arithmetic averages (cluster analysis)
US	Universal sequencing primer
1Bi-1	*P. intermedia* specific oligonucleotide sequence (Dix *et al.*, 1990; Shah *et al.*, 1995)
2Bi-1	*P. nigrescens* specific oligonucleotide sequence (Dix *et al.*, 1990; Shah *et al.*, 1995)
970-11	Arbitrary primer

BACTERIAL NAME ABBREVIATIONS

A.	*Actinomyces; Actinobacillus*
B.	*Bacteroides; Bacillus*
C.	*Capnocytophaga; Campylobacter*
Cl.	*Clostridium*
E.	*Eikenella; Eubacterium*
F.	*Fusobacterium*
G.	*Gemella*
H.	*Haemophilus*
K.	*Kingella*
L.	*Leuconostoc*
Lc.	*Lactococcus*
N.	*Neisseria*
P.	*Porphyromonas; Prevotella; Pseudomonas*
Pstr.	*Peptostreptococcus*
S.	*Streptococcus*
Stom.	*Stomatococcus*
T.	*Treponema*

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THE AUTHOR

Katherine Louise Robertson obtained an upper second class B.Sc. (Hons) degree in Microbiology from the University of Manchester in June 1995. The work within this thesis was completed between September 1995 and September 1998 during which time the author was enrolled in the Department of Plants, Microbes and the Environment within the School of Biological Sciences, University of Manchester. This study was conducted under the supervision of Dr. D. B. Drucker and funded by a MRC Industrial Collaborative Studentship with Colgate-Palmolive Oral Pharmaceuticals. During the three years of this study, the author has had the opportunity to attend and present work in both oral and poster formats at meetings of the Anaerobe Society of the Americas (ASA), British Society for Dental Research (BSDR), International Association for Dental Research (IADR) and the Society of Anaerobic Microbiology (SAM). Details are given below.

Robertson, K.L, Drucker, D.B., Maddocks, L.M., Grady, R. and Davies, R. (1996) RAPD analysis of *Prevotella intermedia* and *Prevotella nigrescens* [Abstract]. Proceedings of the 44th Annual General Meeting, The British Society for Dental Research. *Journal of Dental Research* **75**: 1188. BSDR Abstracts A468.
{poster presentation by KLR at BSDR, Bristol, UK April 1996}

Robertson, K.L, Drucker, D.B. and Blinkhorn, A.S. 1997. RAPD-PCR analysis of *Prevotella intermedia* and *Prevotella nigrescens*. *Journal of Dental Research* **76** (Spec. Iss): 226. IADR Abstracts 1698.
{poster presentation by KLR at IADR, Orlando, USA March 1996}

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{poster presentation by KLR at SAM, Cambridge, July 1996}

Robertson, K.L, Drucker, D.B. and Blinkhorn, A.S. 1998. A comparison of four techniques used to distinguish strains of *Prevotella intermedia* and *Prevotella nigrescens*. Anaerobe (*in press*).
{oral presentation by KLR at ASA, Buenos Aires, Argentina, April 1998}

Robertson, K.L, Drucker, D.B., James, J., Blinkhorn, A.S. and Hamlet, S. 1998. The Microbiology of Papillon-Lefèvre Syndrome. *Journal of Dental Research*. 77 (Spec. Iss. B): 732. IADR Abstracts 803.
{poster presentation by KLR at IADR, Nice, June 1998}

DEDICATION

TO MY DAD

AND

THE LOVING MEMORY OF GRANDAD

1. INTRODUCTION

The microflora of the oral cavity is composed of many species supported and regulated by the complexity of the environment and the interactions of host diet and immune functions. The resident microflora of a healthy mouth is composed of predominantly aerobic species. Environmental flux can disturb the stability of the microflora leading to the development of plaque and the progression to gingivitis and periodontal disease where the composition of the microflora is altered and predominately anaerobic species are found. The influence of medical health on the oral cavity is demonstrated by patients with systemic medical conditions such as Papillon-Lefèvre Syndrome and diabetes mellitus where oral disease is often potentiated. *Prevotella intermedia* and *P. nigrescens* are anaerobic putative periodontopathogens and the role of these organisms in periodontal disease and their phylogenetic relationships is still being explored. Although not life-threatening, the cost of periodontal diseases to the National Health Service in the UK, both in financial terms (over £750 million in 1986-1987; Marsh and Martin, 1992) and in terms of lost working hours, is large. Therefore, research into understanding the causes of periodontal disease is essential.

1.1 THE ORAL CAVITY

1.1.1 ANATOMY OF THE TOOTH

Teeth are composed of a central core of pulp containing the nerve cells and blood supply. This is surrounded by dentine which supports the enamel covering the exposed portion of the tooth. The roots are covered by cementum which is not normally exposed. The structure of a tooth is illustrated in figure 1.1.

1.1.2 THE HEALTHY PERIODONTIUM

Periodontium (Greek; *peri*, around and *odontos*, a tooth; Schroeder and Page, 1990c) is the collective name for the supporting structures of the teeth; gingiva, periodontal ligament, alveolar bone and cementum, a mineralised tissue, similar to bone which covers the root of the tooth (Williams *et al.*, 1992). The periodontium functions to attach the teeth to the bone, protect from the demands of speech and eating and the gingiva provides a barrier protecting underlying tissues from bacterial invasion (Schroeder and Page, 1990c). In a healthy mouth, the gingiva is pale pink in colour, firm and attached to the tooth at the base of the enamel covering (Schroeder and Page, 1990c). A slight protrusion forms the gingival sulcus (also known as gingival crevice or pocket) which is of minimal depth when healthy (Williams *et al.*, 1992). The gingiva is composed of keratinized stratified squamous

epithelium laid over collagen fibres and is attached to the tooth at the amelo-cemental junction, by the junctional epithelium (Williams and Zager, 1978). This part of the gingiva is called the free gingiva. The interdental gingiva assumes a conical shape in the region between teeth and is referred to as the interdental papilla (Schroeder and Page, 1990c) and the attached gingiva which is bound to the alveolar bone and is consistent with the alveolar mucosa lining the mouth (Williams *et al.*, 1992). The periodontal ligament covers the root of the tooth and is embedded in the alveolar bone, suspending the tooth in the socket. It is composed of collagen fibres that allow the slight movements of the tooth as demanded by mastication (Williams *et al.*, 1992). Alveolar bone is consistent with the mandible and maxilla bones of the jaw and surrounds the roots of teeth. If the teeth are lost, this bone will be reabsorbed (Schroeder and Page, 1990c). The basis structure of a tooth and elements of the periodontium is illustrated in figure 1.1.

1.2 ECOLOGY OF THE ORAL CAVITY

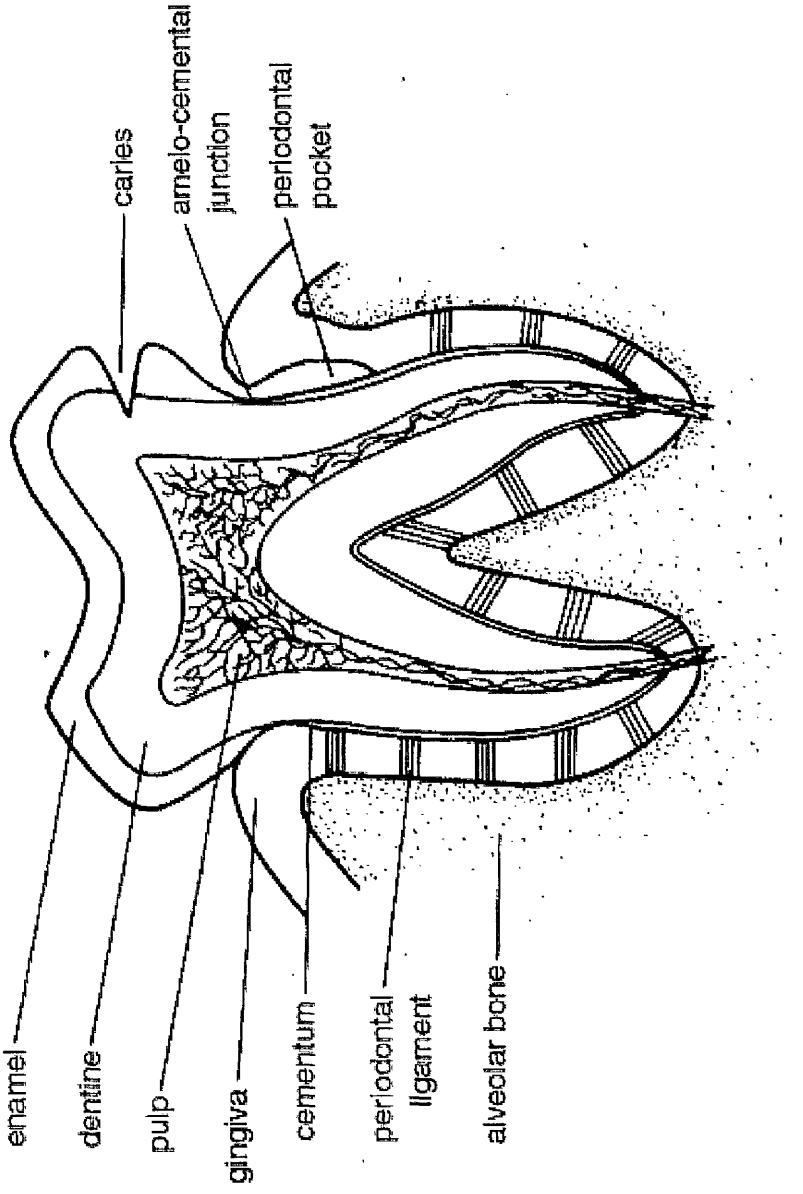
The oral cavity supports the growth of over 350 bacterial species (Moore *et al.*, 1985) as well as fungi belonging to *Candida* species (Dahlén and Wikstöm, 1995). It is a complex environment where unique habitats provide an ecological niche for microbial colonisation and growth. The oral microflora at a given site in the oral cavity, is affected by pH, redox potential (Eh), availability of nutrients and the location of the site of colonisation (Marsh and Martin, 1992).

1.2.1 SITES OF COLONISATION

1.2.1.1 Oral mucosa

The mucosal surfaces of the oral cavity (including cheeks and lips) are colonised soon after birth and remain the only available site for colonisation until the eruption of the primary dentition. The surface of the tongue consists of stratified squamous epithelium. It is covered with tiny projections called papillae that provide a source of shelter for microorganisms from salivary flow and mastication. The tongue also has a low redox potential (Marsh and Martin, 1992) which allows obligate anaerobes to grow under reduced conditions.

Figure 1.1 Structure of a tooth and surrounding tissues



1.2.1.2 Teeth

The eruption of the primary dentition at about three years of age, exposes the enamel, a hard non-shedding surface which is readily colonised. With age, the cementum covering the root may also become exposed and thus colonised. The structure and arrangement of the teeth provides more than one habitat. Smooth surfaces next to the cheek (buccal) or tongue (lingual) afford little protection from saliva flow and are colonised in small numbers. The occlusal (biting surface) contains fissures in addition to the approximal regions between teeth and the gingival crevice that are protected environments which shelter adherent organisms (Marsh and Martin, 1992). The flow of saliva around the teeth provides proteins and free amino acids as potential nutrient sources (see section 1.2.2.4). The initial sites of colonisation are lost with the primary dentition, but replaced at about six years of age when permanent teeth will begin to erupt. Physical changes in the mouth; scaling and polishing, tooth extractions or the addition of prosthetics will cause alterations in the ecological conditions and subsequently in the microorganisms able to exploit the environment (Marsh and Martin, 1992).

1.2.1.3 Gingival crevice

In addition to the tooth surface, eruption of teeth provides another niche, the gingival crevice, also known as a periodontal pocket (Williams *et al.*, 1992) or gingival sulcus (Williams and Zager, 1978). The gingival crevice is found at the point at which the gingiva adheres to the tooth, in a healthy mouth it is absent or a maximum of 2 mm deep (Williams *et al.*, 1992). Gingival crevicular fluid (GCF) flows through the gingival crevice, originating from capillaries in the gingiva and carrying nutrients (see section 1.2.3.2 for details).

1.2.2 FACTORS AFFECTING MICROBIAL GROWTH

The environmental conditions at a given site within the oral cavity will affect the predominant colonising species.

1.2.2.1 Redox (oxidation-reduction) potential (Eh)

The oral cavity contains mostly facultative anaerobic and obligate anaerobic microorganisms as well as micro-aerophilic and capnophilic species, demonstrating a broad spectrum of tolerance to oxygen. Details of bacterial metabolism and the correlation with oxygen requirement are given in section 1.8.1.1, and the potential toxicity of oxygen and the products of its reduction is covered in section 1.8.2.3. The redox potential is the measure

of the oxidation-reduction at a given site (Morris, 1991). The presence of electron donors or reducing agents (e.g. NADH and H_2) which become oxidised, will lower the redox potential. Conversely, electron acceptors or oxidising agents (e.g. oxygen and pyruvate) which become reduced will raise the redox potential. Obligate anaerobes require a low redox potential, or oxidation-reduction level i.e. reduced conditions for their normal metabolism. The presence of oxygen and its use as an electron receptor results in the reduction of oxygen and therefore oxidation of the environment. This raises the redox potential rendering conditions unsuitable for obligate anaerobes. The Eh drops as plaque develops and bacterial species produce carbon dioxide, hydrogen and other volatile fermentation products favouring the growth of anaerobes (cited by Marsh and Martin, 1992).

1.2.2.2 pH

The pH is not constant either throughout the oral cavity or within a specific site. Saliva has a pH in the range 6.75-7.25; this fluctuates constantly. The metabolism of fermentable carbohydrates to lactic acid can cause the pH to drop below pH 5.0 which selects for the aciduric species such as mutans streptococci and lactobacilli (Marsh, 1994) the consequences of which are discussed in section 1.4.1. By contrast, the metabolism of proteins and amino acids raises the pH, for example *Actinomyces viscosus* (cited by Marsh and Martin, 1992) has the ability to convert urea into ammonia (Alfano, 1976). An alkaline pH also accompanies gingival inflammation, with the pH of the GCF rising from pH 6.90 to pH 7.25-7.75 due to the deamination of amino acids and production of ammonia. This pH favours the growth of black-pigmenting anaerobic species (McDermid *et al.*, 1990).

1.2.2.3 Temperature

Bacterial species have adapted to grow over a wide range of temperatures depending upon habitat. The majority of human pathogens and commensals are mesophiles displaying a temperature optimum between 30°C and 40°C. The temperature of the oral cavity is usually 35-36°C allowing the metabolism of many microbial species.

An increase in subgingival temperature is associated with disease and this temperature increase has been shown to be positively correlated with the numbers of putative pathogens; *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* isolated (Haffajee *et al.*, 1992). It has been suggested that the rise

in subgingival temperature is due to the formation of plaque and the subsequent host immune responses (Isogai *et al.*, 1994).

1.2.2.4 Nutrients

Saliva contains a large reservoir of nutrients; proteins, glycoproteins, urea and amino acids which are readily accessible to the majority of habitats within the oral cavity. The GCF carries nutrients such as host proteins and glycoproteins which proteolytic bacteria in subgingival plaque can break down providing amino acids and carbohydrates. Free amino acids and iron-containing molecules such as transferrin, haemopexin, haemoglobin and haptoglobin (Marsh, 1994) are also present. When broken down by black-pigmented anaerobic species (Carlsson *et al.*, 1984) these molecules release haemin which is an essential co-factor for growth. The presence of these nutritional constituents in the GCF helps to explain the differing microflora in the gingival crevice. The intake of additional fermentable carbohydrates through diet (exogenous nutrients) greatly affects plaque microflora (see sections 1.2.2.2, 1.3.1 and 1.4.1).

1.2.3 REGULATION OF MICROBIAL COLONISATION

Microbial colonisation of oral sites is limited by the removal of bacterial cells by host processes. Cells are removed by mastication, swallowing, desquamation of epithelial cells, and the flow of saliva around the mouth and GCF through the gingival crevice. Components of the host immune system are found within GCF and saliva (see sections 1.2.3.2 and 1.2.3.3) and microorganisms must overcome these specific (immune system) and non-specific (saliva flow) host defences in order to proliferate. In addition, antibacterial agents are introduced into the mouth through mouthwashes containing chlorhexidine and toothpastes, many of which contain fluoride which inhibits glycolysis and aids enamel remineralisation (cited by Marsh and Martin, 1992).

1.2.3.1 Inflammatory response

Inflammation is a non-specific response to plaque and bacterial products (Williams *et al.*, 1992). Neutrophils and macrophages migrate to the gingival tissues from the blood where they phagocytose the bacterium and secrete hydrogen peroxide or hypochlorous acid internally to destroy it. They will also secrete enzymes externally to destroy adherent bacteria (Williams *et al.*, 1992) and migrate into the gingival crevice. Macrophages secrete complement components (see section 1.2.3.2 below) which cause opsonisation of bacterial

cells enhanceing phagocytosis and the cytokine interleukin-1 which attracts further inflammatory cells. As well as a cellular response, there is a physiological response. An increased blood flow and dilation of blood vessels allows immune system components from the blood to pass into the tissues (Williams *et al.*, 1992). The subsequent inflammation increases the flow of gingival crevicular fluid as described in section 1.2.3.2 below.

1.2.3.2 Gingival crevicular fluid (GCF)

The flow of GCF through the gingival crevice removes some bacterial cells which are swallowed and the immune system components from the blood within it act to destroy cells. The immunoglobulins IgG, IgM and IgA are present. Complement components circulate causing complement-dependent lysis and both the classical and the alternative complement pathways function (Lehner, 1992a) effecting opsonisation of bacterial cells and attracting cellular mediators of inflammation. The complement reaction is activated via the classical pathway either in response to the formation of bacterial antigen and immune system antibody complexes or via the alternative pathway as a reaction to the cell walls and lipopolysaccharide of plaque bacteria. Bacterial extracellular polysaccharides for example dextran of *Streptococcus mutans* or the lipopolysaccharides of Gram-negative bacterial species will activate the alternative complement pathway. GCF also contains neutrophils, the phagocytic mechanisms of which are enhanced by opsonisation, as well as low numbers of lymphocytes and monocytes.

The flow rate of GCF increases with a rise in gingival inflammation caused by bacterial colonisation and plaque formation (Lehner, 1992a). This raises the levels of immune components and also the levels of nutrients required for bacterial growth.

1.2.3.3 Saliva

Saliva flows throughout the oral cavity, whole saliva is comprised of GCF and fluid from the salivary glands (Lehner, 1992a). The components of saliva function mainly to inhibit adherence of microorganisms and colonisation of surfaces. Saliva contains lysosyme which disintegrates bacterial cell walls by breaking the bond between N-acetyl glucosamine and N-acetyl muramic acid of peptidoglycan, the major cell wall component (Lehner, 1992b). It also contains lactoferrin an iron binding-ligand which competes for free iron. In addition, saliva contains glycoproteins such as mucin which promote aggregation enabling removal by swallowing, as well as sialoperoxidase (cited by Marsh and Martin, 1992) which inhibits the uptake of the amino acid lysine (Lehner, 1992b) through the production of hypothiocyanite

or hypothiocyanous acid in the presence of hydrogen peroxidase (cited by Marsh and Martin, 1992). Hydrogen peroxide is released during the bactericidal activities of phagocytes. Components of the host immune system circulate in the saliva, the predominant antibody is secretory IgA (Marsh, 1989) which agglutinates bacteria and inhibits adherence to surfaces (Lehner, 1992b). Other classes of immunoglobulins can enter the saliva from the GCF. In addition, saliva contains the ions bicarbonate, calcium, chloride, phosphate, potassium and sodium which acts as buffers and help to neutralise the acids produced by bacterial metabolism maintaining a pH typically between 6.75-7.25 (cited by Marsh and Martin, 1992).

1.3 PLAQUE

Dental plaque comprises a mixed microbial community embedded in a polymeric matrix of host salivary glycoproteins, proteins and bacterial polysaccharides. It accumulates on all tooth surfaces and on approximal surfaces is classified as being supragingival or subgingival. The plaque microflora is in an equilibrium state termed microbial homeostasis and this dynamic balance is maintained by antagonistic and synergistic interactions within the heterogeneous population.

1.3.1 THE DEVELOPMENT OF PLAQUE

The development of the plaque biofilm is a dynamic process which is continually in flux. As soon as the teeth are cleaned, salivary glycoproteins and lipids as well as bacterial glucans are adsorbed onto the teeth forming an acquired pellicle (Kolenbrander and London, 1993). The molecules contained within the pellicle enhance bacterial adhesion to the tooth surface (Kolenbrander, 1993). Adherent bacteria may be removed from the tooth surface by brushing but may not be removed from the mouth. Within two hours of cleaning teeth, pioneer species including *Streptococcus sanguis*, *S. mitis*, *S. oralis*, *S. salivarius*, *Neisseria* species, *Actinomyces naeslundii*, *A. viscosus* and *A. israelii* (Slots, 1977) adhere to the pellicle. This adherence depends on mechanisms including lectins (carbohydrate-binding proteins) and glucosyltransferases. Weak electrostatic attractive forces also contribute to initial adherence. The replication of these organisms produces micro-colonies.

Multiplication within the micro-colonies results in confluent bacterial growth called a biofilm. Adherent bacteria may produce extracellular polysaccharides which are important for maintaining the biofilm in its entirety. If the biofilm is not removed by mechanical action, metabolic activity of these species alters the local environment and a microbial

succession to a climax community is seen. In addition secondary colonisers may express adhesins which recognise receptors on primary colonisers facilitating coaggregation amongst oral species (Kolenbrander and London, 1993). Coaggregation may occur between species of one genus (intrageneric), species of different genera (intergeneric) or be multigeneric, as a result of coaggregation to a common partner (Kolenbrander, 1993). The numbers of streptococci reach a maximum within 8 hours (Nyvad and Kilian, 1987). Over time the composition of plaque will alter to include facultative and obligate anaerobes such as *Veillonella* species (Socransky, 1977), *Fusobacterium* (Zee *et al.*, 1996) and *Capnocytophaga* species (Kolenbrander and London, 1993). After 2 weeks plaque consists mainly of the anaerobic bacilli *Porphyromonas* and *Prevotella* species. The late colonisers coaggregate primarily with fusobacteria which serves as a bridge between the primary early colonisers and the later ones. The climax community will vary at different sites within the plaque due not only to coaggregation properties but to concentration gradients of oxygen, carbon dioxide, nutrients and metabolic end-products through the depth of the plaque (Marsh, 1989). The metabolism of the aerobic and facultative anaerobic species colonising the tooth surface, generates carbon dioxide which coupled with a drop in the redox potential (Eh), allows obligate anaerobes to survive. The result is a highly heterogeneous population. Mineral deposition on top of plaque forms calculus (tarter) which is harder to remove than plaque.

1.3.1.1 Supragingival plaque

Supragingival plaque occurs above the gingival margin on the visible tooth surface, its composition is as described above (section 1.3.1). Its accumulation is inhibited mainly by the abrasive action of brushing and by both the flow of saliva and the action of the host defence components it carries, as described in section 1.2.3.3 (Darveau *et al.*, 1997).

1.3.1.2 Subgingival plaque

The accumulation of subgingival plaque (below the gingival margin) is associated with an increased likelihood of periodontal disease (Marsh, 1994). It causes an inflammatory response and increase in the flow of GCF. The increased flow of GCF raises the concentration of nutrients in the gingival crevice, and the increased metabolism creates an alkaline environment as described in section 1.2.2.2. The development of the subgingival plaque is restricted by space (Darveau *et al.*, 1997), although the formation of a gingival pocket as plaque develops, provides a greater area for plaque bacteria to colonise. The

gingival crevice is a protected environment, in terms of saliva flow and mechanical removal but bacterial colonisers must survive the host immune components found within the GCF (Darveau *et al.*, 1997; see section 1.2.3.2 for details). The bacterial species found within subgingival plaque is also determined by the lower oxygen concentrations. Research into the development of subgingival plaque over time, have shown that *S. sanguis* predominates from the time of tooth cleaning, reaching a maximum at 1 day (Socransky *et al.*, 1977). Other facultative anaerobes include members of the *Streptococcus milleri* and *S. oralis* groups (Loesche *et al.*, 1985; Marsh and Martin, 1992). *Actinomyces* species were also common at all stages of development (Socransky *et al.*, 1977), *A. meyeri*, *A. naeslundii* and *A. viscosus* have been found in subgingival plaque (Loesche *et al.*, 1985; Marsh and Martin, 1992). Large numbers of anaerobes including *Veillonella* and *Peptostreptococcus* species have not been detected until 4 days (Socransky *et al.*, 1977). Plaque within the gingival crevice contains a higher numbers of obligately anaerobic species including the black-pigmenting rods *P. melaninogenica* and *P. intermedia*, fusobacteria, *Veillonella* species, spirochaetes and the capnophilic species *Capnocytophaga ochracea* (Loesche *et al.*, 1985; Marsh and Martin, 1992).

1.3.2 MICROBIAL CO-EXISTENCE IN PLAQUE

The formation of microhabitats and concentration gradients of nutrients within the plaque results in a large number of organisms successfully co-existing. As well as the antagonistic relationships caused by nutrient competition and toxic metabolic end product accumulation, mutualistic relationships occur. The enzymes of different species assist in the sequential breakdown of an exogenous nutrient source and many nutritional interactions occur as a result of normal metabolism. *Fusobacterium* (cited by Marsh and Martin, 1992) and *Prevotella* species (Grenier and Mayrand, 1986) release hydrogen and formate, and *Streptococcus* and *Actinomyces* (cited by Marsh and Martin, 1992) release formate, which aid the growth of *Campylobacter* (previously *Wolinella*). *Campylobacter rectus* (*Wolinella recta*) and *Veillonella* (cited by Marsh and Martin, 1992) provide protohaeme and vitamin K respectively stimulating the growth of black-pigmenting bacteria including *P. gingivalis* and *Prevotella* species (Grenier and Mayrand, 1986). Bacterial interaction may also be beneficial to the host, for example *S. sanguis* produces hydrogen peroxide which kills the putative pathogen *A. actinomycetemcomitans* (Socransky and Haffajee, 1992).

1.3.3 BREAKDOWN OF MICROBIAL HOMEOSTASIS

The microbial stability within dental plaque is termed microbial homeostasis (Marsh 1989). A breakdown in microbial homeostasis can occur due to changes in the host immune system, for example AIDS or chemotherapy induced immunosuppression (Marsh, 1989). Environmental factors which include dietary habits alone or in combination with poor oral hygiene result in prolonged episodes of acidic pH (see sections 1.2.2.2 and 1.4.1). The consequence is dental caries or other oral disease.

1.4 ORAL DISEASE

1.4.1 DENTAL CARIES

Dental caries is a significant oral disease which affects large numbers within the population and is an indication of the potentially destructive nature of microbial metabolism. Dental caries is the formation of cavities within the tooth caused by demineralisation of the enamel and destruction of inner tissues by acids. The cariogenic plaque bacteria; lactobacilli (e.g. *L. casei*) and extracellular polysaccharide producing streptococci (e.g. *S. mutans*) metabolise sugars producing acid even at low pH. Acid produced by bacterial metabolism of exogenous and endogenous nutrients lower the pH within the oral cavity, typically below pH 5.0. The hosts diet determines how often the pH is lowered with the frequency increasing with the consumption of between-meal snacks. The low acid conditions select for the aciduric species and increases the risk of dental caries (Marsh, 1994).

1.4.2 GINGIVITIS

Although gingivitis is not strictly a disease of the periodontium, in susceptible individuals it often precedes periodontitis (Williams *et al.*, 1992) and an understanding of it is essential to understanding the pathogenesis of periodontal disease.

1.4.2.1 Chronic marginal gingivitis

Gingivitis is an inflammatory response of the gingiva to an accumulation of dental plaque at the gingival margins (Suzuki, 1988). It is accompanied by an increase in the flow of gingival crevicular fluid (Marsh, 1994). The disease is characterised by redness and swelling of the gingiva and a tendency to bleed on probing. The gingiva may be sensitive and itchy (Suzuki, 1988) with no loss of periodontal support. Gingivitis often precedes periodontitis (Dibart, 1997). It is reversible (Tanner and Stillman, 1993) with the establishment of good oral hygiene procedures.

1.4.2.2 Acute necrotizing ulcerative gingivitis (ANUG)

This is an acute form of gingivitis that is thought to be stress related (Shannon *et al.*, 1969) and aggravated by smoking (Walter and Shields, 1977). It is characterised by inflamed, painful, bleeding gingiva and ulceration of interdental papillae leading to necrosis (Walter and Shields, 1977) or blunting (Johnson and Engel, 1986) over which a grey pseudomembrane is formed (Suzuki, 1988). The patient suffers from halitosis (Johnson and Engel, 1986).

1.4.2.3 Other forms of gingivitis

Other forms of gingivitis can occur as a result of hormonal changes during steroid therapy or increases in the levels of oestrogens and progesterones during pregnancy and puberty or in women taking oral contraceptives (Suzuki, 1988). These hormones stimulate the synthesis of prostaglandins, which mediate inflammation, as well as providing bacterial growth factors (Dougherty and Slots, 1993). Gingivitis can be seen as manifestations of other diseases, such as HIV-associated gingivitis in HIV-positive patients (Tanner and Stillman, 1993) and acute herpetic gingivostomatitis which is an infection by the virus Herpes simplex type I (Dougherty and Slots, 1993). Taking certain medication, such as cyclosporin and phenytoin has also been associated with the gingivitis symptoms of swelling, inflammation and pain (Suzuki, 1988), this may prevent adequate oral hygiene and allow gingivitis to occur.

1.4.3 PERIODONTAL DISEASE

The general term periodontal disease or periodontitis is used to encompass a number of conditions which have very similar clinical presentations (Schroeder and Page, 1990a). All exhibit plaque-induced inflammation of periodontal tissues and affect the supporting structures of the teeth, the periodontal ligament is destroyed, alveolar bone reabsorbed and the junctional epithelium undergoes apical migration down the root surface, deepening the gingival crevice to form what is commonly called a periodontal pocket (Williams, 1990). Collectively, this is described as loss of attachment and this destruction is irreversible.

1.4.4 CLASSIFICATION OF PERIODONTAL DISEASE

There is some confusion in the literature regarding the classification of types of periodontal disease and the terminology assigned to them. The descriptions below are based on the guidelines of the World Workshop in Clinical Periodontology 1989 (American Academy of

Periodontology, 1989; cited by Williams *et al.*, 1992), and summarised in table 1.1. Despite guidelines, overlap still exists between classifications (Attström and van der Velden, 1994), especially generalised juvenile periodontitis and rapidly progressive periodontitis and the classification of periodontitis associated with systemic diseases (American Academy of Periodontology, 1989). The European Workshop on Periodontology (Attström and van der Velden, 1994) suggests a simpler approach restricted to three categories. These are (i) early onset periodontitis, (ii) adult periodontitis and (iii) necrotizing periodontitis with classifications based on aetiology and host response (Attström and van der Velden, 1994) and therefore considering the periodontal components of systemic disease separately.

Table 1.1 Classification of periodontal disease based on the report of the World Workshop in Periodontology in 1988 (American Academy of Periodontology, 1989)

GINGIVITIS
Chronic marginal gingivitis
Acute necrotizing ulcerative gingivitis (ANUG)
GINGIVITIS ASSOCIATED WITH OTHER FACTORS
Hormones: pregnancy, puberty, oral contraceptive pill
GINGIVITIS ASSOCIATED WITH SYSTEMIC DISEASE
HIV-associated gingivitis (HIV-G)
Acute herpetic gingivostomatitis
Drug induced gingival overgrowth: phenytoin, cyclosporin
PERIODONTITIS
Chronic adult periodontitis
Acute necrotizing ulcerative periodontitis (ANUP)
Early onset periodontitis
Juvenile periodontitis: localised juvenile periodontitis (LJP)
generalised juvenile periodontitis
Rapidly progressive periodontitis
Pre-pubertal periodontitis: generalised pre-pubertal periodontitis
localised pre-pubertal periodontitis
PERIODONTITIS ASSOCIATED WITH SYSTEMIC DISEASE
Insulin-dependent diabetes mellitus
Papillon-Lefèvre Syndrome
Downs Syndrome
Neutropenia
Hypophosphatsia
Histocytosis X
Leukaemia
Leukocyte adhesion deficiency
HIV-associated periodontitis (HIV-P)

Adapted from Williams *et al.* (1992).

1.4.4.1 Chronic adult periodontitis

This is the common form of periodontitis seen within the adult population. The term is usually used for patients over 30 years of age (Williams *et al.*, 1992). There are high levels of dental plaque consistent with the severity of disease (Page and Schroeder, 1982). Inflammation of the gingiva and gingival recession is seen. Loss of attachment occurs but it is unclear whether it is continual, increasing in a linear fashion (Jeffcoat and Reddy, 1991) or random, occurring in bursts separated by periods of disease inactivity (Goodson *et al.*, 1982). In mild cases plaque removal is effective treatment and tooth loss does not occur (Williams, 1990). Advanced cases are treated by root planing and surgery (Williams, 1990).

1.4.4.2 Early onset periodontitis

This term covers periodontal disease seen in young people under the age of 35 years (American Academy of Periodontology, 1989). Periodontal breakdown occurs as previously described but has a much earlier onset and rapid progression and only low levels of plaque are seen (Williams *et al.*, 1992). Juvenile periodontitis, pre-pubertal periodontitis and rapidly progressive periodontitis are described below.

1.4.4.2.1 Pre-pubertal periodontitis

Periodontal breakdown occurs after eruption of the primary dentition (Williams, 1990), affects both dentitions (Williams *et al.*, 1992) and usually affects children under 14, typically between the ages of 5 and 8 years (Suzuki, 1988). The disease can occur in localised or generalised forms (Suzuki, 1988). The occurrence is rare and has associated leukocyte abnormalities (American Academy of Periodontology, 1989). Generalised forms may be accompanied by other infections including those of skin and upper respiratory tract (Suzuki, 1988). Papillon-Lefèvre Syndrome (PLS; see section 1.4.5.1) is an example of a genetic skin disease, sometimes with associated neutrophil dysfunction and the oral presentations of pre-pubertal periodontitis (D'Angelo *et al.*, 1992). The classification of periodontitis associated with systemic disease is unresolved (American Academy of Periodontology, 1989).

Generalised pre-pubertal periodontitis

Generalised pre-pubertal periodontitis is the most acute form and is usually associated with systemic disease (Dougherty and Slots, 1993), especially leukocyte adhesion deficiency (Watanabe, 1990). It affects all of the primary dentition and may progress to affect some or

all of the permanent teeth (Page and Schroeder, 1982). There is severe inflammation of the gingiva accompanied by spontaneous haemorrhage and cleft formation (Page and Schroeder, 1982). Periodontal breakdown is rapid and severe (Page and Schroeder, 1982) and conventional therapy; mechanical plaque removal and antibiotic treatment, is ineffective (Tinanoff *et al.*, 1986) and the teeth are often extracted (D'Angelo *et al.*, 1992; Tinanoff *et al.*, 1986).

Localised pre-pubertal periodontitis

Localised pre-pubertal periodontitis affects only some of the deciduous teeth (Page and Schroeder, 1982). It progresses at a slower rate than the generalised form and may fail to exhibit inflamed gingiva (Page and Schroeder, 1982). Some patients show no evidence of systemic disease (Dougherty and Slots, 1993). Defining cases as localised pre-pubertal periodontitis has been problematic and it has been suggested that there is a type which involves all teeth and occurs in the absence of leukocyte adhesion deficiency, such as is found in patients with PLS and hypophosphatasia (Watanabe, 1990). The American Academy of Periodontology (1989) published a paper (cited by Watanabe, 1990) to the effect that systemic diseases like PLS, neutropenia, hypophosphatasia and agranulocytosis were not included under the definition of pre-pubertal periodontitis. These conditions are mentioned in section 1.4.5, periodontitis associated with systemic disease.

1.4.4.2.2 Juvenile periodontitis

This type of periodontitis has an onset around puberty (Williams, 1990) affecting otherwise healthy (Baer, 1971) patients aged 12 to 26 years (Suzuki, 1988), there is little plaque (Page and Schroeder, 1982) and limited inflammation but rapid periodontal destruction (Williams, 1990). The onset may be due to an increase in the concentration of hormones in the blood and GCF serving as growth factors for bacteria (Schroeder and Page, 1990a). Generalised and localised forms have been described.

Localised juvenile periodontitis (LJP)

LJP is characterised by low levels of dental plaque (Listgarten, 1976). The initial disease progression is rapid but slows over time (Baer, 1971). It typically affects the first permanent molars and incisor teeth (Williams *et al.*, 1992) and has been described in patients presenting no clinical symptoms of underlying systemic conditions (Butler, 1969). A family tendency to LJP is seen (Baer, 1971) and more females are affected than males

(Page and Schroeder, 1982). LJP may progress to generalised juvenile periodontitis (Williams *et al.*, 1992) or gradually slow progression to a halt (Page and Schroeder, 1982).

Generalised juvenile periodontitis (GJP)

LJP may progress to affect the entire dentition with molars and first incisors affected most severely (American Academy of Periodontology, 1989).

1.4.4.2.3 Rapidly progressive periodontitis

Rapidly progressive periodontitis typically occurs after 20 years of age (Williams *et al.*, 1992) up to 35 years of age (Page and Schroeder, 1982), although it can start at puberty (Page and Schroeder, 1982). It has a similar clinical presentation to LJP however all teeth can be affected and as a result it is also described as generalised juvenile periodontitis (Williams *et al.*, 1992), however the onset is typically later in life (American Academy of Periodontology, 1989). The disease progresses in cycles of severe, rapid periodontal destruction with gingiva that are inflamed and bleeding, pus formation from periodontal pockets may also be seen (Page and Schroeder, 1982). This is followed by a periods of inactivity when an absence of inflammation is noted (Williams *et al.*, 1992).

1.4.5 PERIODONTITIS ASSOCIATED WITH SYSTEMIC DISEASE

There are a number of systemic conditions known to accelerate or increase the amount of periodontal destruction. Frequently, patients with insulin-dependent (Mendieta and Reeve, 1993) and non-insulin-dependent diabetes mellitus (Emrich *et al.*, 1991) suffer from severe rapidly progressive periodontitis (Gottsegen, 1990) which may be complicated by abscesses. The control of diabetes correlates with lessening symptoms (Dougherty and Slots, 1993). Patients suffering from leukaemia's have gingival enlargement due to infiltration of leukaemic cells into the gingiva (Dougherty and Slots, 1993). A form of generalised periodontitis affects patients with neutrophil functional defects; leukocyte adhesion deficiency and a reduction in numbers; neutropenia (Dougherty and Slots, 1993). Genetically inherited diseases with associated host immune system defects *viz.* Down's Syndrome and Chediak-Higashi Syndrome (Williams, 1995) and patients with Papillon-Lefèvre Syndrome (Dougherty and Slots, 1993; see section 1.4.5.1) exhibit severe periodontitis. The oral manifestations of hypophosphatasia are a lack of cementum formation (Williams, 1995) and rapid exfoliation of the deciduous dentition (Dougherty and Slots, 1993), occasionally teeth with large pulp chambers (Dougherty and Slots, 1993) are

seen. Ascorbic acid deficiency (Williams *et al.*, 1992), HIV infection (Dougherty and Slots, 1993), inflammatory bowel disease and Histocytosis X (Mendieta and Reeve, 1993) also have periodontal symptoms.

1.4.5.1 Description of Papillon-Lefèvre Syndrome

Papillon-Lefèvre Syndrome (PLS) was first described in 1924 by Papillon and Lefevre. It is genetically inherited as an autosomal recessive disease (Griffiths *et al.*, 1992) which means that both of a child's parents must carry the gene. It has been suggested that consanguinity between parents is seen in one-third of cases (Hattab *et al.*, 1995) and affected siblings are sometimes seen (Gorlin *et al.*, 1964). PLS is rare with an incidence of 1-4 per million people (Gorlin *et al.*, 1964). The disease is characterised by hyperkeratosis which is a redness and thickening of the skin (Griffiths *et al.*, 1992), which may appear dry, scaly and cracked (Hart and Shapira, 1994). This typically occurs on the palms and the soles of the feet, often extending to the Achilles tendon (Gorlin *et al.*, 1964). Called palmar-plantar (Gorlin *et al.*, 1964) or palmoplantar (Hart and Shapira, 1994) hyperkeratosis, it can also be seen on elbows and legs (Hattab *et al.*, 1995). It can appear in childhood as early as 2 months old (Brown *et al.*, 1993) and may lessen in severity with increasing age (Haneke, 1979). Seasonal fluctuations in the severity of palmar-plantar hyperkeratosis have been reported (Hart and Shapira, 1994). In addition, precocious periodontal destruction of both the deciduous and permanent teeth is seen (Haneke, 1979), which is illustrated in figure 1.2. The loss of teeth is the most constant feature and often that which leads to the diagnosis of PLS (Haneke, 1979). However, cases of PLS displaying unaffected primary teeth (Brown *et al.*, 1993; Bullon *et al.*, 1993; Rateitschak-Plüss and Schroeder, 1984) or mild periodontal destruction (Brown *et al.*, 1993) have been reported. An absence of palmar-plantar hyperkeratosis in a child with the periodontal component and a family history of siblings with both components of PLS (Soskolne *et al.*, 1996) has also been reported. The periodontal manifestations become apparent after the uneventful eruption of the normal primary dentition (Haneke, 1979). The gingiva become red and inflamed and may be ulcerated (Posteraro, 1992) and a form of rapid, severe periodontitis occurs (Hattab *et al.*, 1995). Pus is formed so that the mouth has a fetid odour (Hattab *et al.*, 1995). Alveolar bone is reabsorped and deep periodontal pockets form leading to increased tooth mobility (Haneke, 1979), as the alveolar support decreases the patient has difficulty chewing (Hattab *et al.*, 1995). Ultimately, all deciduous teeth are lost prematurely around the age of 4-5 years old (Micali *et al.*, 1994) and the periodontal symptoms disappear (Haneke, 1979).

Although the permanent teeth appear normal before eruption and erupt without event, the cycle repeats itself (Haneke, 1979). All teeth are usually lost by the age of 13-15 years old (Hattab *et al.*, 1995). Teeth are exfoliated in the same order that they erupt (Micali *et al.*, 1994) although the third molars are sometimes spared (Gorlin *et al.*, 1964). Late onset cases have been reported with patients in the age range 20-30 years old (Brown *et al.*, 1993). Patients may exhibit other clinical symptoms including dermatological abscesses, osteoporosis, mental retardation and intercranial calcifications (Micali *et al.*, 1994). An increased susceptibility to infection has also been reported (Haneke *et al.*, 1975), including furunculosis (Haneke, 1979), spastic bronchitis and conjunctivitis (D'Angelo *et al.*, 1992).

Reports regarding the treatment of the periodontal component of PLS are variable so that antibiotic treatment has been reported as a failure (Rateitschak-Plüss and Schroeder, 1984) as well as successful in combination with mechanical therapy and extraction of the primary teeth (Tinanoff *et al.*, 1986).

A clinically similar syndrome, in which patients exhibit palmoplantar hyperkeratosis, severe periodontal destruction coupled with elongation of the bones of the hands and the feet (arachnodactyly) has also been described (Haim and Munk, 1965).

1.4.5.2 Aetiology of Papillon-Lefèvre Syndrome

Many theories on the aetiology of PLS have been proposed which can be divided into anatomical, host response and bacterial (French *et al.*, 1995). Anatomical and host response theories will be dealt with briefly below and the bacterial aetiology is covered in more detail in section 1.7.

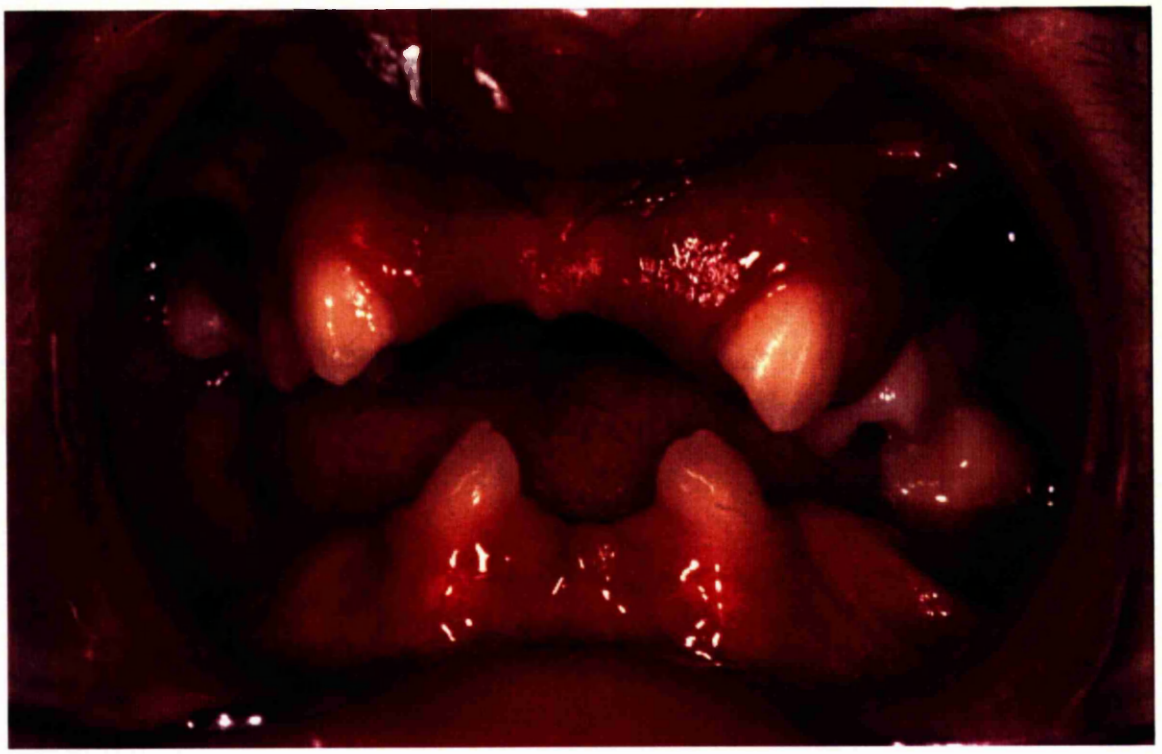
Anatomical

Changes to the gingival epithelium like those seen on the palmar-plantar regions have been suggested (Lyberg, 1982), damage to the epithelium would compromise the integrity of the epithelial barrier and allow bacteria to enter tissues underneath (Page, 1986). This would enhance the normal host response to plaque and exacerbate the consequences. Keratin abnormalities have been proposed (Aso *et al.*, 1987 cited by Hart *et al.*, 1997) in PLS patients, mutations in the keratin proteins may upset differentiation and alignment of keratin fibres and subsequently the structure of epithelial cells (Hart *et al.*, 1997). Reduced cementum thickening (Vrahopoulos *et al.*, 1988) and altered collagenetic activity (Shoshan *et al.*, 1970) have also been reported.

Host response

There is evidence for a compromised host immune response in PLS sufferers. Although many studies have reported decreased neutrophil chemotaxis (Tinanoff *et al.*, 1986; Van Dyke *et al.*, 1984) and phagocytosis (Djawari, 1978), other researchers have not found this (Lyberg, 1982; Rateitschak-Plüss and Schroeder, 1984). However, a return to normal neutrophil function has been reported with resolution of periodontal infection (Bullon *et al.*, 1993; Tinanoff *et al.*, 1986) and it has been suggested that neutrophil deficiencies are a result of disease rather than a cause (Bullon *et al.*, 1993). Information regarding the production of superoxide radicals is equally controversial because both increases (Bimstein *et al.*, 1990) and decreases in their production by polymorphonuclear leukocytes have been reported (Bullon *et al.*, 1993).

Figure 1.2 Typical oral presentation of a patient with Papillon-Lefèvre Syndrome



1.5 RISK MARKERS FOR PERIODONTAL DISEASE

High risk categories for the development of destructive periodontal disease consist of many interacting determinants which are difficult to separate (Wolff *et al.*, 1994). Periodontal disease has many associated risk factors (Beck, 1994), but broad separations into environmental, host related and bacterial factors can be made with interactions between these groupings becoming apparent (Wolff *et al.*, 1994).

1.5.1 ENVIRONMENTAL FACTORS

Poor oral hygiene and infrequent dental visits are associated with increased plaque levels, inflammation and GCF flow (Wolff *et al.*, 1994) increasing the risk of disease. Smoking has an adverse affect on the immune system (Haber *et al.*, 1993). The local vasoconstrictive effect of nicotine lessens blood flow to the gingiva, reducing the circulation of components of the immune system in these areas, resulting in impaired immunity (Rivera-Hidalgo, 1986). It also exerts a systemic effect reducing antibody production (Haber *et al.*, 1993). Chemotactic and phagocytic functions of polymorphonuclear leukocytes (PMNs) are depressed by tobacco smoke metabolites (Rivera-Hidalgo, 1986) and less gingival inflammation is seen (Bergstrom and Preber, 1994). MacFarlane *et al.* (1992) conducted a study of PMN defects in patients with refractory periodontitis (that is patients with adult periodontitis that fails to respond to treatment), and found PMN phagocytosis impaired. In addition, 90% of the patients smoked, increasing their risk of developing destructive periodontal disease. Smokers have been shown to have a statistically greater odds of having an increased periodontal pocket depth compared to non-smokers (Stolenberg *et al.*, 1993). Whilst a deeper periodontal pocket depth was associated with the presence of certain putative periodontopathogens including *P. intermedia* and *A. actinomycetemcomitans*; smoking itself showed no significant correlation with the occurrence of these organisms (Stolenberg *et al.*, 1993) confirming earlier work by Preber *et al.* (1992) which described how the proportions of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* where unchanged in smokers and non-smokers. Decreased immunity caused by smoking, plus an increased periodontal pocket depth with associated putative periodontal pathogens results in a high risk of periodontal disease development.

Stress is thought to be a predisposing factor in the pathogenesis of periodontal disease (Linden *et al.*, 1996) and ANUG (Shannon *et al.*, 1969). Periods of stress have affects on the autonomic nervous system and endocrine functions (Walter and Shields, 1977). Corticosteroids and catecholamine are secreted, providing growth factors for

microorganisms, including *P. intermedia* (Horning and Cohen, 1995). The release of vasoconstrictors epinephrine by the adrenal medulla and norepinephrine (Walter and Shields, 1977) causes reduced blood flow to the gingival tissues and decreased immune function.

An individual reaction to stress causes changes in dietary habits (see section 1.4.1 dental caries), oral hygiene and smoking (Walter and Shields, 1977) which contribute to periodontal disease as previously described in this section.

1.5.2 HOST FACTORS

Older patients, persons of Afro-Caribbean origin and those with a lower education level exhibit a greater prevalence and extent of periodontitis among an American population (Oliver *et al.*, 1991). A study of a Sri Lankan population with no access to dental care showed calculus formation at a very early age with extensive coverage by the mid-twenties (Ånerud *et al.*, 1991). In contrast, a Norwegian population with good access to dental care did not show calculus formation until the age of 20 years at the earliest (Ånerud *et al.*, 1991) so that a lack of education and poor oral hygiene can be seen to be important contributory factors.

Diabetes acts as an accelerating factor for periodontitis, possibly due to either increased blood glucose levels or an immune defect (Gottsegen, 1990). A study (Haber *et al.*, 1993) of smoking habits of insulin-dependent diabetes mellitus patients and non-diabetic patients concluded that although diabetic patients had a greater prevalence of more severe periodontal disease, the correlation between smoking and periodontal disease was stronger.

Diet and nutrition also have a role in periodontal health (Alfano, 1976). The components of diet, providing endogenous nutrients for oral cavity dwelling microorganisms influences the pH of the oral cavity as described for carbohydrate protein intake in section 1.2.3.1. In turn, pH influences the microflora and the state of health (see section 1.4.1). Malnutrition lowers the phagocytic capabilities of leukocytes (Alfano, 1976) which increases risk of bacterial infection and subsequent disease (Wolff *et al.*, 1994) and vitamin C deficiency causes scurvy which exacerbates the destructive effects of microbial plaque (Schroeder and Page, 1990b).

1.5.3 BACTERIAL FACTORS

Many bacterial species can be isolated from disease sites in the oral cavity (see section 1.6 below) although it is not often clear whether they or not they have an aetiological role

(Wolff *et al.*, 1994). The natural development of plaque, exacerbated by poor oral hygiene procedures will increase the amount of gingival inflammation and the rate of GCF flow (see section 1.3 above for details of plaque accumulation). As a result the proportions of the anaerobic putative pathogens (see section 1.6 below for details of microflora associated with disease) will increase (Wolff *et al.*, 1994).

The occurrence of particular microorganisms within the mouth is associated with an increased risk of periodontal destruction (Haffajee *et al.*, 1991). Haffajee *et al.* (1991) suggested that increased levels of *P. gingivalis*, *P. intermedia* and *P. micros* but decreased levels of *Capnocytophaga* species coupled with an increased subgingival temperature resulted in increased risk of periodontal destruction. However a two and a half year study by Listgarten *et al.* (1991) reported that neither the presence nor absence of *P. intermedia* (and *A. actinomycetemcomitans*) could be used as a disease indicator. This suggests that the mere presence of a periodontopathogen was not enough to cause disease (Socransky and Haffajee, 1992) and even if the pathogen was present, it must be in a favourable environment in a susceptible host (Socransky and Haffajee, 1992).

1.6 MICROBIAL AETIOLOGY OF ORAL DISEASE

The subgingival microflora in a healthy mouth consists mostly of Gram-positive anaerobic and facultative rods belonging to the genus *Actinomyces* and facultative Gram-positive streptococci (Slots, 1977). The species isolated alters with progression from healthy to diseased sites. Table 1.2 is adapted from Darveau *et al.* (1997) and lists species found in subgingival plaque, associated with health and disease.

Table 1.2 Some bacterial species found in subgingival plaque, associated with the oral cavity in health and disease

HEALTHY ORAL CAVITY	
Streptococcus oralis, S sanguis, S. mitis, S. gordonii,	
S. mutans, S. anginosus, S. intermedius,,	
Gemella morbillorum	
Rothia dentocariosa	
Actinomyces naeslundii, A. gerencseriae, A. odontolyticus	
Peptostreptococcus micros	
Eubacterium nodatum	
Capnocytophaga ochracea, C. gingivalis	(also gingivitis)
Campylobacter gracilis	
Fusobacterium nucleatum subsp. *polymorphum*	
GINGIVITIS	
S. oralis, S. sanguis, S. mitis, S. intermedius	
C. ochracea, C. gingivalis	
C. gracilis	
Prevotella loescheii	
Pstr. micros	
E. nodatum	
A. naeslundii, A. israellii	
Campylobacter concisus	
A. odontolyticus	
Fusobacterium nucleatum subsp. *nucleatum*	
E. brachy	
Eikenella corrodens	
Actinobacillus actinomycetemcomitans (serotype a)	
PERIODONTITIS	
Porphyromonas gingivalis	(also gingivitis)
Actinobacillus actinomycetemcomitans (serotype b)	
Bacteroides forsythus	(also gingivitis)
Spirochaetes	
Treponema denticola	
P. intermedia	(also gingivitis)
P. nigrescens	(also gingivitis)
Campylobacter rectus	(also gingivitis)
Pstr. micros	(also gingivitis)
Fusobacterium nucleatum subsp. *vincentii*	
Fusobacterium nucleatum subsp. *nucleatum*	
Selenomonas noxia	
Selenomonas flueggeii	(also gingivitis)
Enteric species	
Fusobacterium alocis	
Lactobacilli uli	(also gingivitis)
Veillonella parvula	(also gingivitis)

Adapted from Darveau *et al.* (1997). Species listed in descending order of association.

1.6.1 GINGIVITIS

1.6.1.1 Chronic gingivitis

Many species such as *Eubacterium brachy, Streptococcus anginosus, A. naeslundii, S. sanguis, Campylobacter concisus, Peptostreptococcus anaerobius, Treponema pectinovorum* and *T. socranskii* (Moore *et al.*, 1987) have been isolated from sites of gingivitis in both adults and children. Other species including *Capnocytophaga ochracea* and *Propionibacterium acnes* have been isolated more frequently from children than adults, whilst *Eubacterium nodatum, F. alocis, S. morbillorum* and *Veillonella parvula* were more numerous in adults (Moore *et al.*, 1987).

1.6.1.2 ANUG

P. intermedia, Fusobacterium, Treponema and *Selenomonas* species have been isolated from patients with ANUG (Loesche *et al.*, 1982).

1.6.2 PERIODONTITIS

The microorganisms involved in periodontal disease are predominantly, although not exclusively Gram-negative anaerobic rods (Dzink *et al.*, 1985).

1.6.2.1 Adult periodontitis

Studies of periodontal lesions have isolated *Fusobacterium nucleatum, A. actinomycetemcomitans, P. gingivalis, P. intermedia, B. forsythus, C. rectus, Peptostreptococcus micros* (Dzink *et al.*, 1988; Savitt and Socransky, 1984), *E. nodatum, E. timidum, F. alocis, P. anaerobius* (Moore *et al.*, 1991) and *Selenomonas sputigena* (Haffajee *et al.*, 1984) from actively progressing sites and *C. ochracea, Streptococcus mitis, S. sanguis* and *Veillonella parvula* from inactive sites (Dzink *et al.*, 1988). A pathogenic role for black-pigmenting anaerobic rods has been suggested for many years (Spiegel *et al.*, 1979; White and Mayrand, 1981). Strong associations between *P. gingivalis* and *P. intermedia* (as well as *A. actinomycetemcomitans*) have been reported by Slots *et al.* (1986) who detected at least one of these organisms in 99% of the active lesions sampled and these species are now widely accepted as putative periodontopathogens (Dahlén, 1993; Slots, 1986a). The presence of spirochaetes (Loesche *et al.*, 1985; Savitt and Socransky, 1984) including *Treponema pectinovorum, T. socranskii* (Moore *et al.*, 1991) and *T. denticola* (Riviere *et al.*, 1992) has also been recorded.

1.6.2.2 Juvenile periodontitis

Actinobacillus actinomycetemcomitans has been associated with LJP (Savitt and Socransky, 1984; Zambon *et al.*, 1983) but not in all cases (Loesche *et al.*, 1985). *C. ochracea* has also been isolated (Savitt and Socransky, 1984) and a recent study detected high levels of *P. gingivalis* and *P. intermedia* in samples from patients with LJP and GJP (López *et al.*, 1996).

1.6.2.3 Prepubertal periodontitis

The following organisms have been isolated from sites of localised pre-pubertal periodontitis, *F. nucleatum*, *P. intermedia*, *A. actinomycetemcomitans*, *Capnocytophaga sputigena* and *E. corrodens* (Delaney and Kornman, 1987).

1.7 MICROBIAL AETIOLOGY OF PAPILLON-LEFÈVRE SYNDROME

Microbiological studies of the oral microflora of Papillon-Lefèvre Syndrome patients have shown that the predominant organisms in the periodontal sites are Gram-negative anaerobic (Newman *et al.*, 1977) and facultative rods (Ishikawa *et al.*, 1994), spirochaetes have also been isolated (Ishikawa *et al.*, 1994; Rateitschak-Plüss and Schroeder, 1984). Species isolated include *P. gingivalis*, *Prevotella intermedia*, *P. loescheii*, *Bacteroides gracilis* (Clerehugh *et al.*, 1996), *Eikenella corrodens*, *F. nucleatum* (Clerehugh *et al.*, 1996; Tinanoff *et al* 1986), *Capnocytophaga* (Tinanoff *et al.*, 1986), *Veillonella* and anaerobic streptococci (Eronat *et al.*, 1993), not unlike studies of adult periodontitis (Dzink *et al.*, 1988; Slots *et al.*, 1986). The presence of cultivatable *A. actinomycetemcomitans* or raised serum antibody levels has been reported in many cases (Bimstein *et al.*, 1990; Clerehugh *et al.*, 1996; Eronat *et al.*, 1993; Ishikawa *et al.*, 1994; Van Dyke *et al.*, 1984), but not universally (D'Angelo *et al.*, 1992; Tinanoff *et al.*, 1986). This suggests an important association between *A. actinomycetemcomitans* and PLS (Preus and Gjermo, 1987) and it has been speculated that its presence is vital for tooth loss. It has been hypothesised that the periodontal component is due to infection rather than a symptom of disease (Preus, 1988). This species has been linked to another destructive periodontal disease of children, LJP (Page and Schroeder, 1982; Zambon *et al.*, 1983).

Scanning electron microscopy studies of plaque determined that the supragingival plaque consists mainly of cocci and fusiform bacteria (Jung *et al.*, 1981) and the subgingival plaque contains spirochaetes (Jung *et al.*, 1981).

1.8 DETECTION AND IDENTIFICATION OF PUTATIVE PERIODONTO-PATHOGENS IN CLINICAL SAMPLES

The detection, identification and classification of the microorganisms involved in periodontal disease is important. From a clinical perspective it enables the microbiologist to determine the causative organisms of disease and to study the commensal organisms present. Assessments can be made determining individuals in a high risk group for developing periodontal disease (Watanabe and Frommel, 1996). The study of strain genotypes is useful to find out whether disease is caused by limited number of strains in all patients or within a particular patient group (Eisenstein, 1990) and to study the transmission of organisms for example within families (Preus *et al.*, 1994). Identification of the cause of clinical infection means effective treatment (Watanabe and Frommel, 1996). From a taxonomic viewpoint it allows continued understanding of the relationships between bacterial species and improves knowledge regarding individual species and from a practical perspective there is continued development of new techniques and refinement of others.

Although the terms classification and identification are used closely, an isolate must be classified and given an identity before it can be identified (Ludwig and Schleifer, 1994). Whilst some of the techniques described in this section have a role in the characterisation and classification of organisms, it is beyond the scope of this work which focuses primarily on the use for detection and identification.

Texts dedicated in aiding the clinical microbiologist identify medically important species provide identification tables and detailed characteristics of individual species. The *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) provides detailed information regarding the identification of anaerobic bacterial species. It details three levels of identification; I, based purely on Gram stain and colony morphology; II additional tests for catalase, nitrate, indole and lipase production and antibiotic susceptibilities to kanamycin, vancomycin and colistin determined and level III, the use of miniaturised test kits (API, RapID ANA) and Gas-Liquid Chromatography (GLC). Typical GLC traces can be found in the *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977). Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) provides identification tables for Gram-positive and Gram-negative bacteria. The first stage identification tables are based on cell shape, spore formation (Gram-positive isolates only), motility, growth aerobically and anaerobically, catalase and oxidase production, acid production from glucose metabolism and breakdown of carbohydrates. Second and third stage tables are provided giving more specific information for the particular genera including nitrate and nitrite reduction,

haemolysis on blood agar plates, pigment formation, temperature tolerance and growth or failure to grow on specific media. Colony types from a mixed inoculum must be subcultured to obtain pure culture (Collins *et al.*, 1995). This is essential before further characterisation work can be performed.

1.8.1 SELECTIVE MEDIA AND CULTURE

A general purpose media can be used to grow and isolate as many bacteria as possible from a sample. Selective media enhance the chances of isolating specific species (Cowan and Steel, 1993). The addition of antibiotics to a medium will inhibit unwanted organisms and select for those species resistant to it, for example kanamycin-vancomycin laked blood agar selects for *Prevotella* and *Bacteroides* species (Summanen *et al.*, 1993), the kanamycin inhibits most Gram-negative rods including fusobacteria (Wren, 1991) and vancomycin inhibits Gram-positive organisms. Selective agents can also be added to inhibit certain species, for example facultative Gram-negative rods and swarming clostridia (*C. septicum*) are inhibited by phenylethyl alcohol sheep blood agar (Summanen *et al.*, 1993). Media may also be chosen which relies on characteristics of the organism desired like the bile resistant nature of the *B. fragilis* group and *Bilophila* with the use of Bacteroides bile esculin agar (Summanen *et al.*, 1993) which contains 20% bile to inhibit anaerobes and gentamicin to inhibit aerobes. Incubation of clinical specimens in both anaerobic and aerobic environments is pertinent as the oxygen tolerance of a species is useful for identification.

1.8.1.1 Oxygen Requirements - Aerobiosis and Anaerobiosis

Species of bacteria differ in their tolerance of oxygen (see section 1.8.4.1 on bacterial metabolism), a factor which is useful for identification (see section 1.8.1.2 and 1.8.1.3). OBLIGATE AEROBES (e.g. *Bacillus* sp.) require oxygen for aerobic respiration and are unable to undergo fermentation. Aerobic organisms can process the toxic metabolites of oxygen (superoxide ion O_2^- and hydrogen peroxide H_2O_2) to harmless water and oxygen through enzymatic reactions (see section 1.8.2.3.1 below for more details). In contrast, OBLIGATE ANAEROBES (e.g. *Fusobacterium* sp.), grow only in the absence of oxygen, lacking the ability to undergo aerobic respiration or process the toxic by products of oxygen metabolism. A FACULTATIVE anaerobe is an aerobic organism which utilises environmental oxygen when present but can adapt to anaerobic metabolism in its absence. AEROTOLERANT anaerobes (e.g. streptococci) will survive in the presence of oxygen, having the ability to breakdown the toxic metabolites but do not utilise it, their metabolism remains

fermentative. MICROAEROPHILIC organisms such as *Actinomyces* sp. require a small amount of oxygen. Microbes requiring increased carbon-dioxide tensions are termed CAPNOPHILIC.

1.8.1.2 Aerobic culture

Medical specimens should be incubated in an aerobic environment between 35°C and 37°C (Collins *et al.*, 1995).

1.8.1.3 Anaerobic culture

There are two widely used methods to generate anaerobic environments.

1.8.1.3.1 Anaerobic jars

Airtight jars provide an anaerobic environment after the catalytic removal of oxygen (Cowan and Steel, 1993) as it combines with hydrogen on the surface of palladium pellets. Commercial sachets (Oxoid Ltd) are readily available to produce the gaseous requirements of hydrogen (Wren, 1991) or hydrogen-carbon-dioxide (Collins *et al.*, 1995) after the addition of water. The catalyst is deactivated by the water produced when oxygen and hydrogen combine, but it can be reactivated by heating to 160°C for at least two hours (Summanen *et al.*, 1993). Anaerobic conditions are obtained in several hours, confirmed by condensation. The entire jar is incubated at the desired temperature.

1.8.1.3.2 Anaerobic cabinets

Anaerobic cabinets may be used as incubators or workstations (Collins *et al.*, 1995), offering permanent anaerobic conditions which allows culture plates to be inspected without removing them from an anaerobic environment (Summanen *et al.*, 1993). Entry into the cabinet is via glove ports, which may be purged with anaerobic gases to remove oxygen, many models have an airlock which allows equipment to be transferred into the cabinet (Cowan and Steel, 1993). The anaerobic gases is a mixture of 5-10% H_2, 5-10% CO_2 and 80-90% N_2 (Summanen *et al.*, 1993). Anaerobic cabinets also contain catalysts, the life of which is prolonged by the inclusion of Anotox® (Don Whitley, West Yorks, UK). This is an activated charcoal which adsorbs gases such as hydrogen sulphide (produced by bacterial metabolism of sulphur containing media; Cowan and Steel, 1993) which poison the catalyst irreversibly (Wren, 1991).

1.8.2 TRADITIONAL LABORATORY TESTS

A pure culture may be examined in several ways.

1.8.2.1 Gram stain

The Gram stain is the sequential application of dyes, crystal-violet, iodine and safranin to a biofilm of bacteria. The cells of Gram-positive and Gram-negative bacteria stain a different colour which is attributable to differences in the structure of the cell wall between the organisms *viz.* peptidoglycan and outer membrane of Gram-negative cells. The thin peptidoglycan layer of the Gram-negative cell takes up less dye (crystal-violet and iodine complex) than the thick peptidoglycan layer of Gram-positive cell walls. The application of acetone dissolves the outer membrane of Gram-negative cells allowing the crystal-violet and iodine complex to be removed allowing cells to take up the counterstain, safranin which stains them red. The crystal-violet and iodine complex is retained by the peptidoglycan of Gram-positive cells staining them purple. The Gram stain result will help determine which commercial identification kit (see section 1.8.3 below) is required and the shape, size and orientation of cells will also help to establish tentative family identifications, although the morphology is affected by the cultural conditions and the medium used (Cowan and Steel, 1993). The Gram stain should always be performed on a young culture as the cells which stain Gram-positive loose the ability to resist decolourisation with acetone as they age and will therefore stain Gram-negative (Cowan and Steel, 1993). This is not the only problem with the Gram stain. In addition, members of the genus *Mobiluncus* are Gram-negative cells which appear to acquire Gram-positivity with age and members of the genus *Gemella* are Gram-positive cocci which are easily discoloured and therefore often reported as being Gram-negative (Cowan and Steel, 1993).

1.8.2.2 Colonial characteristics

Colony morphology should also be examined as certain periodontopathogens have distinct colony types. Examples are *Actinomyces israelii* which has a distinctive molar tooth shaped colony, breadcrumb like colonies are suggestive of *Fusobacterium nucleatum* (Summanen *et al.*, 1993) and *Eikenella corrodens* (Cowan and Steel, 1993) and *Bacteroides ureolyticus* (Summanen *et al.*, 1993) which form pits in the agar surface. Details of the colony morphology of many anaerobic species can be found in print (Summanen *et al.*, 1993). The colony colour is particularly useful for some species. Light tan through brown to black suggests *Prevotella* and *Porphyromonas* species (Summanen *et al.*, 1993) although

Peptostreptococcus niger also produces black pigment (Wren, 1991) and *Bilophila* species are black on Bacteroides bile esculin agar (Summanen *et al.*, 1993). Pink to red is indicative of *A. odontolyticus* (Summanen *et al.*, 1993) and *Capnocytophaga* species are often yellow or pink (Leadbetter *et al.*, 1979). It must be remembered that the type and extent of haemolysis will be affected by the type of blood used in agar plates (Stokes *et al.*, 1993).

1.8.2.3 Other tests

There are many tests that can be performed to identify bacterial isolates and texts such as Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) provide comprehensive information. The three tests that were most commonly used during this study are described below, these and other tests are discussed in the discussion, section 4.11.

1.8.2.3.1 Catalase test

The catalase test detects the ability of the test organism to breakdown hydrogen peroxide (H_2O_2) to oxygen and water via the enzyme catalase (equation 1). Hydrogen peroxide is a toxic oxygen intermediate which may be present in a bacterium as a result of the incomplete reduction of oxygen and the conversion of superoxide ions (O_2^-) plus hydrogen by the enzyme Superoxide Dismutase (equation 2). The oxygen intermediates, plus the hydroxyl free radical ($^{\bullet}OH$) which can be formed by H_2O_2 plus O_2^- (equation 3 the Haber-Weiss reaction; Morris, 1991) damage all cellular structures including proteins, membranes and DNA, and are released by phagocytic cells of the human immune system. In the diagnostic test, this action is demonstrated by the production of oxygen seen as bubbles.

Equation 1: $2H_2O_2 \longrightarrow 2H_2O + O_2$

Equation 2: $O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

Equation 3: $O_2^- + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + OH^-$

1.8.2.3.2 Oxidase test

The oxidase test detects the presence of Cytochrome c Oxidase which oxidises oxygen by the addition of electrons from hydrogen, to form water. In the diagnostic test, this action is demonstrated the development of colour.

1.8.2.3.3 Oxidation-fermentation test

The oxidation-fermentation test determines whether an organism degrades carbohydrates by oxidation or fermentation (Hugh and Leifson, 1953). Each organism to be tested is grown in two tubes of medium containing an indicator, one is covered with a layer of paraffin to exclude air from the medium. The indicator bromothymol blue is used which turns from blue at pH 7.0 to yellow at pH 6.0. Oxidisers will breakdown carbohydrate in the open tube only, displaying a yellow colour but for fermenters a yellow colour is seen for both the open and the paraffin overlaid tubes (Hugh and Leifson, 1953).

1.8.3 API (APPAREILS ET PROCEDES D' IDENTIFICATION) COMMERCIAL IDENTIFICATION KITS

API (bioMérieux, France) identification systems consist of plastic strips containing a series of wells or cupules which contain dehydrated test substrates for the detection of performed enzymes. Strips are inoculated with a suspension of the test organism and incubated in an aerobic environment according to the manufacturer's instructions. Reactions are identified directly by spontaneous colour changes or by the addition of reagents. The pattern of colour changes is converted into a numerical code, which is used to identify the organism based on a comparison of characters with those of other organisms in a reference database (Busse *et al.*, 1996). The identification is accompanied by a percentage probability. Identification systems are available for anaerobes, coryneform bacteria, staphylococci, streptococci, enterobacteria, non-enteric Gram-negative rods and yeasts. The reactions within API miniaturised enzymatic tests are described in appendix 6.15.

Other miniaturised identification systems are available and a comprehensive list is given by Busse *et al.* (1996). The use of two other systems; Minitek system (Becton Dickinson) and Vitek system (Vitek Inc., USA) is discussed in relation to the identification of anaerobic organisms in the *Wadsworth Anaerobic Laboratory Manual* (Summamen *et al.*, 1993).

1.8.4 GAS-LIQUID CHROMATOGRAPHY

Gas-Liquid Chromatography (GLC) has been used as a chemotaxonomical tool by analysing the long chain fatty acid composition of microbial cells and for the identification of microorganisms by the analysis of fermentation end-products (Drucker, 1981). Fermentation allows the production of energy without the need for oxygen, this metabolism by anaerobic bacteria yields acids and alcohol that are removed from the cell into the surrounding medium (Drucker, 1981). There are different fermentation types each resulting

in the formation of different products and certain groups of bacteria utilise particular pathways (Drucker, 1981). The use of this characteristic for bacterial detection and identification was first proposed in 1966 (Henis *et al.*) when it was noted that bacterial strains could be distinguished by assessing the presence or absence of particular peaks. This characteristic is genetically stable (Summanen *et al.*, 1993), and tables listing fermentation end-products of anaerobic bacteria (Summanen *et al.*, 1993) as well as sample profiles (Holdeman *et al.*, 1977) have been produced to allow the analysis of end-products to be used to aid identification. The amount and type of end-products can be influenced by the liquid medium in which an organism is grown as well as the time of incubation, therefore standardisation is essential. In a medium which contains glucose and peptone, glucose will be metabolised first giving high yield of glucose-specific end-products before peptone is fermented. Peptone rich media will result in greater amounts of *iso*-acids produced (Summanen *et al.*, 1993). End-products accumulate over time and affected by the medium as described above, may appear to alter for the same species, therefore the incubation time must be standardised and the growth medium defined (Drucker, 1981). Microorganisms to be analysed are grown in broth. Before use, the purity is determined as contaminating species may alter the end-product profile (Henis *et al.*, 1966). The cells are removed (Drucker, 1981) to allow analysis of the culture supernatant.

1.8.4.1 Bacterial Metabolism

Bacterial metabolism is divided into anabolism, which is the synthesis of structures and cellular components, and catabolism, which is the breakdown of fuels to release energy. There are three basic catabolic pathways; aerobic respiration, anaerobic respiration and fermentation which are coupled to the oxygen requirement (described in section 1.8.1.1) of a particular species. An overview of each process is provided below.

1.8.4.1.1 Aerobic respiration

Aerobic respiration is undertaken by some aerobic bacteria and involves a series of enzyme-catalysed reactions during which glucose is broken down via glycolysis and the tricarboxylic acid (TCA) cycle. The electrons released are passed along the respiratory chain yielding ATP, until being finally accepted by oxygen.

Glycolysis (or the Embden-Meyerhof pathway) which occurs in the cell cytoplasm, is a series of steps during which glucose is phosphorylated, (a process which consumes 2 ATPs) before being oxidised to pyruvate (2 molecules for every 1 molecule of glucose) through a

series of intermediates. This process yields 2 ATPs and 1 NADH. Pyruvate is cycled through the TCA cycle (also in the cell cytoplasm) where its consecutive conversion into organic acids and ultimately carbon-dioxide yields 1 ATP, 4 NADH and 1 $FADH_2$ per molecule of pyruvate. The last stage is the flow of electrons from the reduced carriers (NADH and $FADH_2$) that accepted electrons during glycolysis and the TCA cycle, this occurs in the cell membrane. The electrons are passed from NADH through 6 carriers in a series of redox reactions, the energy released is used to produce ATP yielding a total of 3 ATP per NADH or 2 ATP per $FADH_2$, a total of 34 ATP. The terminal electron acceptor is oxygen, which is reduced to water. The potential dangers of incomplete reduction of oxygen are discussed in section 1.8.2.3.1. Aerobic respiration yields a net total of 38 ATP.

1.8.4.1.2 Anaerobic respiration

The process of anaerobic respiration involves the same pathways described for aerobic respiration, but alternative final electron acceptors are used instead of oxygen. Inorganic ions such as sulphate (Morris, 1991) or the nitrate reductase system which removes oxygen from nitrate forming nitrite ions (Drucker, 1981). Anaerobic respiration is as efficient as aerobic respiration in terms of ATP yield.

1.8.4.1.3 Fermentation

Organisms which produce energy by fermentation utilise glycolysis to oxidise incompletely carbohydrates to pyruvate. Oxygen and other inorganic electron acceptors are not required for fermentation and the yield of ATP is lower than with other forms of energy producing metabolism. Pyruvate can then be fermented in numerous ways and the exact organic end-products are dependent on the bacterial species allowing the detection to be used for identification. Fermentation types can be broadly grouped as alcoholic or acidic. Three examples of fermentation are; (a) lactic acid fermentation in which pyruvate is reduced to lactic acid by streptococci and lactobacilli, (b) mixed acid fermentation in which acetic and formic acids are produced and (c) ethanolic fermentation which produces ethanol and water (Drucker, 1981).

1.8.4.2 Theory of Gas-Liquid Chromatography

The basic mechanism of a gas chromatograph is as follows. A moving carrier gas phase (usually oxygen-free nitrogen) passes through pressure or flow controllers to the column which is situated within an oven, and is at a pre-programmed temperature. A stationary

liquid phase is absorbed onto the column packing which maintains proportions of gas:liquid. The test sample dissolved in solvent is injected into the beginning of the column and the temperature of the injector head ensures that the sample will vaporise. Separation of substances within the sample occurs during elution through the column. Some substances will tend to remain in the mobile gas phase whilst other molecules will tend to be dissolved in the stationary phase. The constant flow of carrier gas removes molecules in the vapour phase causing molecules to leave the liquid phase in order to restore equilibrium. A detector at the end of the column detects substances as they are eluted and this feeds an electric current to an amplifier and then to a recording device which produces a peak (Drucker, 1981). The volume of carrier gas required to elute a substance is called the retention volume, whilst the length of time a taken for a substance to be separated and eluted is the retention time (Drucker, 1981). Different solutes will be eluted from the column at different times according to their molecular weight and polarity (Summanen *et al.*, 1993), allowing the components of a mixture to be separated and identified by comparison of the retention time with that of known standards (Drucker, 1981).

1.8.4.3 Sample

The samples are generally injected onto the column in 1-10 µl volumes, they may be gas, solid or liquid. The injection is carried out through a silicone rubber septum which must be checked regularly as leaks may increase retention times (Drucker, 1981).

1.8.4.4 Column

There are two types of column; a packed column where the stationary phase coats an inert, thermally stable supporting material or capillary columns where the stationary phase coats the inside of the column. The stationary phase is very important to the separation, it must be inert and pure and is chosen to have a polarity suited to sample, to ensure the solubility of the sample within the stationary phase (Drucker, 1981).

1.8.4.5 Temperature

A sufficiently high temperature is required to ensure that all components of a mixture are volatile. However, a temperature which is too high leads to column bleeding and the production of a background signal as the volatility of the stationary phase is increased (Drucker, 1981).

1.8.4.6 Detector systems

A commonly used detector is a flame ionisation detector (FID) which works by burning the eluted substances in an air-hydrogen flame and the flow of ions is measured by electrodes (Drucker, 1981). An FID detector can detect very small quantities of substances and it is not affected by the presence of water in the samples (Drucker, 1981). Formic acid is not detected by the FID detector, where the detection of formic acid is required an alternative type of detector must be used. Holdeman *et al.* (1977) used a thermal conductivity detector (TCD) to construct the fermentation end-product profiles in the *Anaerobe Laboratory Manual*. The TCD requires only the carrier gas, instead of carrier gas plus air and hydrogen as required by the FID and is sensitive to water present in samples (Summanen *et al.*, 1993). The flame photometric detector burns samples like the FID but measures the intensity or the temperature of the flame (Drucker, 1981). Radioactive ionisation detectors are also available (Drucker, 1981).

1.8.4.7 Application

GLC has several applications useful for the identification of bacterial species. Microbial cells can be examined for the relative proportions of structural components such as lipids as well as by pyrolysis-GLC which is the thermal breakdown of molecules into smaller more volatile ones. Full, informative details of these techniques can be found elsewhere (Drucker, 1981).

The most commonly used application is the analysis of the end-products of fermentative metabolism. This may be done using culture supernatants, from which volatile fatty acids can be detected directly by GLC but methyl derivatives of non-volatile standards must be prepared (Summamen *et al.*, 1993). Head-space gas analysis has also been used by sampling the vapour phase of a culture (Drucker, 1981).

Fermentation end product analysis has been used to classify oral black-pigmenting anaerobes including *P. gingivalis*, *P. melaninogenica*, *P. loescheii* and *P. denticola* (Takada and Hirasawa, 1997).

1.8.5 POLYMERASE CHAIN REACTION (PCR)

1.8.5.1 Theory

The polymerase chain reaction (PCR) was first described by Kary Mullis (Mullis and Faloona, 1987). It is a technique used to amplify specific or target sequences of DNA using primers which are designed based on DNA sequence information for the desired region

(Mullis and Faloona, 1987). PCR consists of repetitive cycles of three defined temperature changes (Saiki, 1989). Within the controlled temperature changes the defined reaction components which are oligonucleotide primers, deoxynucleotide triphosphates, enzyme in a suitable buffer with magnesium ions, synthesise DNA strands (Saiki, 1989). Each cycle of the PCR reaction occurs in three steps. Firstly, template denaturation where the double stranded template is denatured to single stranded DNA at a high temperature, usually within the range 92-96°C. This is followed by primer annealing. The temperature is lowered to allow the primers to anneal to complementary regions of the single stranded DNA. An excess of primers ensures efficient annealing (Mullis and Faloona, 1987). The annealing temperature is chosen to be less than the theoretical melting temperature (T_m) of the primers and often falls in the range 37-65°C. The annealing step should be optimised for specificity as a high temperature prevents mispriming and the amplification of non-specific products. The final step is extension of the product. A further temperature change to an intermediate temperature (72-74°C), promotes the synthesis of the new DNA strand. A heat stable DNA polymerase (*Taq*) adds free deoxynucleotide triphosphates (dNTPs) onto the oligonucleotide primers to produce double stranded DNA molecules extending in the 5' - 3' direction. If the target sequence is particularly long, the length of this step may be increased. A longer extension step may be added to the final cycle to ensure complete strand synthesis. The number of cycles performed is variable, it is usually 25-35 cycles after which time there is negligible increase in the amount of product.

With each cycle of amplification the number of fragments of target DNA increases exponentially, because primer extension products from one cycle serve as templates in the next cycle. This exponential amplification is not immediate, during the first cycle, extension stops once the temperature is raised (94°C) producing long products. The second cycle results in products with assorted lengths (Mullis and Faloona, 1987). Long products are produced in a linear fashion (Mullis and Faloona, 1987) from the extension of primed template DNA and shorter products produced from the extension of the long products produced in the first cycle. In subsequent cycles, short products (of desired length) are produced from long ones and from existing short products, these now accumulate exponentially and act as templates for the primers (Mullis and Faloona, 1987). The amount of enzyme becomes a limiting factor in the accumulation of amplified product. This is an automated process which occurs in a thermal cycler, thus there is potential for numerous samples to be run consecutively. The temperature is strictly controlled to give uniform heating and cooling of all samples regardless of position within the wells of the thermal

cycler. Strict control is also exerted over the ramping time (the time taken to change temperature), as this may affect the efficiency of the cycles and as a result the amplification. The sensitive nature of PCR means that the procedure must be undertaken with the utmost care. Any DNA present has the potential to be amplified if by chance it happens to contain complimentary binding sites. Potential sources of contamination are; cross contamination of template DNA, exogenous DNA from laboratory environment, skin, hair or contamination with previous PCR products (Van Belkum and Niesters, 1995). PCR set-up should be undertaken in a designated area (Van Belkum and Niesters, 1995), and the use of positive displacement filter pipette tips to avoid aerosols (Engleberg, 1994) and aliquotting small volumes of reagents for separate use and storage will help minimise the risks (Smith *et al.*, 1995). Every PCR reaction must include a negative control which checks for contamination of the reaction with either target DNA or exogenous DNA resulting in a false-positive result (Lo *et al.*, 1988). Positive controls are also important as they show reliability and reproducibility of results when performed at least twice.

1.8.5.2 PCR Reagents

A PCR reaction requires the following components; oligonucleotide primers, magnesium ions, free deoxynucleotide triphosphates (dNTPs), a thermostable enzyme and template DNA. In addition, ultrapure water is required for dilutions, controls and to make up volumes.

1.8.5.2.1 Primers

PCR utilises two oligonucleotide primers designed to be complementary to the 5-prime ends of the DNA strands of desired sequence (Simon *et al.*, 1991). The primers flank this sequence on opposite strands of DNA and the region between them is amplified. When the PCR primers are annealed to the template, the enzyme is able to catalyse the production of the oligonucleotide chains. PCR primers are designed to be in the range of 15-30 nucleotides long with a base composition which is approximately equal in all 4 bases (Williams, 1989) and similar melting points (Roux, 1995). The structure is critical, avoiding complementary regions or a complicated secondary structure. The primers must not be able to form hairpin loops, therefore palindromic sequences within primers are to be avoided (Simon *et al.*, 1991) and primer pairs must not have complementary 3-prime ends which encourages primer-dimer formation (Williams, 1989). Primer-dimers are artefacts of the PCR reaction and can be described as a double stranded molecule with a length close to that

of both primer which is formed by the action of polymerase on overlapping primers (Saiki, 1989). They are efficient templates and will predominate in a PCR (Saiki, 1989). Primer-dimers can be also be seen when there are low quantities of template (Saiki, 1989). *Taq* has no exonuclease activity in the 3′ to 5′ direction, therefore it is desirable that the last few bases are an exact match to the target (Simon *et al.*, 1991). It is undesirable for the primers to be able to anneal with themselves or to regions of DNA other than the desired one. The amount of primer used must be carefully optimised. Too much encourages primer dimer formation as well as other non-specific amplification products and too little may result in reduced product yield.

1.8.5.2.2 Enzyme

A range of thermostable enzymes are available which catalyse the production of polynucleotides from an oligonucleotide primer. The enzyme adds monodeoxynucleoside triphosphates in a 5' to 3' direction at a rate in the range of 35-100 nucleotides per second at 70-80°C, with a processivity (the number of nucleotides added before the enzyme dissociates from the template) of 75 nucleotides. The enzyme commonly used in PCR reactions is the thermostable DNA polymerase called *Taq* polymerase from the bacterium *Thermus aquaticus* (Mullis, 1990). Recombinant alternatives are also available, such as *AmpliTaq* a modified version expressed in *E. coli*. Other natural DNA polymerases include Vent DNA polymerase from *Thermococcus litoralis*, Hot *Tub*™ from *Thermus flavis* and *Pfu* from *Pyrococcus furiosus*. *Taq* has optimum activity at 72°C and this is exploited in the extension step. The enzyme is active at the high temperatures of the denaturation step although it has a half life of 40 min at 95°C (Gefland, 1989). Another type of DNA polymerase is the Stoffel fragment, a derivative of *Taq* it has amino acids deleted from the N terminus. It exhibits greater thermostability and is useful if an increased denaturation temperature is required for GC rich regions.

The enzyme used is chosen based upon individual requirements, for example Vent DNA polymerase has a lower misincorporation rate due to 3′-5′ nuclease activity.

1.8.5.2.3 Magnesium ions

The efficiency of the amplification reaction depends critically upon the magnesium concentration because it is an essential cofactor for the enzymatic activity of DNA polymerases and enhances primer/template interactions (Welsh and McClelland, 1991). The

free concentration is affected by buffer type used and the concentration of dNTPs, which chelate magnesium as it is essential for their incorporation (Roux, 1995). Therefore altering the concentration of either of these variables necessitates repeated magnesium titration. A concentration of magnesium which is too high will prevent the complete denaturation of double stranded DNA, while one that is too low may inhibit extension (Saiki, 1989). The optimum chosen is that which provided the clearest amplicons after gel electrophoresis.

1.8.5.2.4 Deoxynucleotide triphosphates (dNTPs)

Production of an oligonucleotide chain from a primer, by an enzyme is due to the addition of dNTPs. Each is composed of a phosphate, a pentose sugar and a nitrogen base. All four nitrogen bases (purines; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate and pyrimidines; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate) are present in equal concentrations which is related to the concentration of magnesium ions (as described above), if the concentration of dNTPs is too low, the synthesis rate of *Taq* decreases (Gefland, 1989).

1.8.5.2.5 Template DNA

The template DNA must be free from impurities which are known to inhibit the PCR reaction these include magnesium ion chelating agents and heavy metals such as iron and detergents. Theoretically, a single copy of the target sequence is all that is required for successful PCR, however, multiple copies increases the likelihood of success.

1.8.5.2.6 Mineral Oil

A layer of mineral oil is overlaid on the reaction mixture to prevent evaporation of reaction mixture during the temperature cycling and to help maintain temperatures (Williams, 1989). This layer is not always necessary, dependent on the thermal cycler machine used.

1.8.5.3 PCR based techniques

There are many variations of the basic PCR technique all aimed at increasing the yield and specificity of the reaction.

Variations include HOT START PCR which is useful were the target is present in low copy numbers, the reaction components are heated to 94°C to denature the template, prior to the addition of *Taq* to minimise non-specific annealing. MULTIPLEX PCR utilises a combination of 2 or more primer pairs to amplify several targets, allowing the detection of multiple PCR

products. PCR WITH DEGENERATE PRIMERS allows amplification of protein coding regions and uncloned genes by designing primers using protein sequence information and combinations of primers matching conserved amino acid sequence. QUANTITATIVE PCR is low cycle number PCR designed to give exponential amplification only, to compare target amounts with known amounts of standards, amplified over a range of concentrations to produce a standard curve (Atlas and Bej, 1994). RANDOM AMPLIFICATION OF POLYMORPHIC DNA BY POLYMERASE CHAIN REACTION (RAPD-PCR) is another variation, which is discussed in detail in section 1.8.6.

1.8.5.4 Application of PCR

Specific PCR primers have been designed to *P. gingivalis*, *Treponema denticola* and *A. actinomycetemcomitans* (Watanabe and Frommel, 1996). Others based on 16S rRNA sequences have been designed to *B. forsythus*, *Campylobacter rectus*, *Eikenella corrodens* (Slots *et al.*, 1995), *P. intermedia*, *P. nigrescens*, *C. sputigena* and *C. ochracea* (Conrads *et al.*, 1996) and have been used with varying degrees of success to detect these organisms in plaque samples.

1.8.5.5 The theory of gel electrophoresis

The visualisation of DNA molecules or fragments of DNA by gel electrophoresis is the end point of the PCR based techniques described in sections 1.8.5 (Polymerase Chain Reaction), 1.8.6 (Random Amplification of Polymorphic DNA by PCR), 1.8.8 (PCR-sequence analysis) and RFLP described in section 1.11.10. Nucleic acids are negatively charged, therefore when placed in an electric field they will migrate towards the positive electrode (Martin, 1996). If they are placed into a gel (usually made of agarose, a long chain polysaccharide, or polyacrylamide) containing a matrix of pores through which the DNA must migrate then the movement depends on size, and charge. Due to the fact that the charge of all DNA molecules is the same, the separation is achieved due to their size and the resistance of the medium determined by pore size. Controlling the pore size, determined by the concentration of agarose will allow molecules of desired sizes to be separated. In other words, gel electrophoresis allows the separation of DNA molecules by size with smaller molecules travelling faster than larger ones (Martin, 1996). Electrophoresis is performed under a buffer which is highly conductive and also compensates for any changes to the pH (Martin, 1996). Nucleic acids are detected after electrophoresis usually by staining with ethidium bromide which intercalates in the double stranded molecule and fluoresces,

fluorescence and radioactivity as well as direct detection by nucleic acid probe may also be used (Martin, 1996). Gels stained with ethidium bromide can be photographed by placing on a transilluminator and using an orange (Wratten 22) filter for optimum contrast (Martin, 1996).

1.8.6 RANDOM AMPLIFICATION OF POLYMORPHIC DNA BY POLYMERASE CHAIN REACTION (RAPD-PCR)

1.8.6.1 Theory of RAPD-PCR

Random amplification of polymorphic DNA by polymerase chain reaction (also known as arbitrarily primed PCR; AP-PCR), is a technique which was first described by Welsh and McClelland (1990) and Williams *et al.* (1990). Unlike PCR it utilises a single oligonucleotide primer which is shorter in comparison to those used in conventional PCR, having an optimum length of 9-10 bases (Williams *et al.*, 1990). The sequence is chosen at random where no previous genomic sequence information is known (Welsh and McClelland, 1990) but the rules concerning structure and sequence orientation apply, as described in section 1.8.5.2. Low stringency conditions are used in order to amplify fragments of DNA by RAPD-PCR, allowing mismatches (Welsh and McClelland, 1990). Sites within the genome with complementarity to the primer occur at random (Muralidharan and Wakeland, 1993) and therefore, the profile of amplification products is dependant on the primer used and the genomic DNA (Welsh and McClelland, 1990). Electrophoretic separation of the DNA fragments produces strain or species-specific profiles and highlights genomic differences. Mutations (insertions or deletions) within the genome will affect the amplification profile (Muralidharan and Wakeland, 1993). These genomic polymorphisms act as genetic markers for the construction of genetic maps and successful characterisation of strains.

1.8.6.2 RAPD-PCR compared with PCR

RAPD-PCR reactions are set up in an identical way to conventional PCR although a higher concentration of primer and therefore magnesium is often used. Defined temperature changes are used and the main difference between the thermal cycles employed for PCR and RAPD-PCR can be seen at the annealing step. For PCR reactions the primers are designed to be complimentary to regions flanking the target and annealing is specific. High stringency conditions ensure that only complimentary regions hybridise so that only the desired sequence is amplified. RAPD-PCR uses primers of arbitrary sequence hence it is

unknown whether or not there will be any regions of complementarity. Lower temperature conditions mean a decreased specificity which will allow base mismatches in the primer annealing. The differences between RAPD-PCR and PCR are listed in table 1.3.

1.8.6.3 Application of RAPD-PCR

RAPD-PCR has been used to study the clonal diversity of isolates of *P. gingivalis* (Ménard and Mouton, 1995) *F. nucleatum* (George *et al.*, 1997) and differentiate strains of *A. actinomycetemcomitans* (Preus *et al.*, 1993). It has also been used to study the transmission of *P. gingivalis* (van Steenbergen *et al.*, 1993) and *A. actinomycetemcomitans* (Preus *et al.*, 1994) and *F. nucleatum* (George *et al.*, 1997).

Table 1.3 Comparison of differences between RAPD-PCR and PCR

RAPD-PCR	PCR
First described by Welsh and McClelland (1990) and Williams et al. (1990)	First described by Kary Mullis (Mullis and Faloona, 1987)
Primers short, often of length 9-10 bases	Primers longer, length 18-25 bases
Primers of arbitrary sequence	Primers of specific sequence
Requires no knowledge of DNA sequence information	Requires knowledge of DNA sequence of region of interest
Utilises low stringency annealing step, allows primer mismatches to occur	Utilises high stringency annealing step, specific primer annealing occurs
Random amplification occurs	Selective amplification occurs

1.8.7 NUCLEIC ACID PROBES

1.8.7.1 Theory

The presence of unique nucleic acid sequences in all organisms (Albandar and Olsen, 1990) and the ability of single-stranded nucleic acid to bind complementary regions (Savitt *et al.*, 1990) is the basis of nucleic acid probes. Sequences chosen can be strain specific or highly conserved and found in all strains of a species (Tenover, 1988). There are three types of probe; whole genomic which consist of the entire genomic sequence and may lack specificity, cloned and oligonucleotide probes which are much shorter (Savitt *et al.*, 1990). Sequences can also been chosen in the absence of molecular information based on RAPD-PCR fragments (Van Belkum and Niesters, 1995). The chosen single stranded sequences are reproduced, labelled and used to hybridise with the complementary strand of the single stranded target (Albandar and Olsen, 1990), which has been immobilised on a support (Savitt *et al.*, 1990). Many types of label are in use, including radioactivity (French *et al.*, 1986), fluorescent dyes (Schleifer *et al.*, 1993), enzymes, antibodies or colourmetric substrates (Tenover, 1988). The specificity of the reaction is controlled by pH, temperature and salt concentration (Tenover, 1988) to prevent non-specific hybridization (Albandar and Olsen, 1990), and unbound probe is removed by washing before detecting bound probe by exposure to radiographic film (Savitt *et al.*, 1990) or fluorescence microscopy (Amann *et al.*, 1990). Short oligonucleotide probes are less sensitive than whole genomic or cloned probes but do have the advantage of greater specificity (Albandar and Olsen, 1990). The sensitivity of nucleic acid probes is greater than cultural based techniques (Savitt *et al.*, 1990). As well as DNA, Ribosomal RNA can be used as a target for nucleic acid probes (Schleifer *et al.*, 1993) due to reasons detailed in section 1.8.8.3 and the high copy number meaning higher sensitivity (Schleifer *et al.*, 1993).

1.8.7.2 Application

The use of nucleic acid probes allows identification of microorganisms direct from clinical samples without the need for cultivation, meaning fastidious organisms (Albandar and Olsen, 1990) including oral spirochaetes (DiRienzo *et al.*, 1991) can be detected. Over a decade, nucleic acid probes against *A. actinomycetemcomitans*, *P. gingivalis* (French *et al.*, 1986), *Wolinella* (*Campylobacter*) *recta*, *F. nucleatum*, *E. corrodens* (Lippke *et al.*, 1989), *B. forsythus* (Lotufo *et al.*, 1994), *P. intermedia* and *P. nigrescens* (Conrads and Brauner, 1995) and *Capnocytophaga* species (Conrads and Brauner, 1995) have been tested.

1.8.8 RIBOSOMAL RNA (RRNA) SEQUENCE ANALYSIS

1.8.8.1 Theory

The development of PCR based techniques and the ability to amplify conserved regions of ribosomal rRNA (rRNA) of taxonomic value, has lead to the development of PCR-sequence analysis (Engleberg, 1994). Using primers to conserved regions (Engleberg, 1994), rRNA genes can be amplified to obtain rDNA (Busse *et al.*, 1996) and sequenced using forward and reverse primers chosen according the length of gene to be sequenced (Busse *et al.*, 1996). Universal primers (to conserved regions) are well cited in the literature. There are primer pairs for the amplification of nearly full length rRNA (Lane, 1991) as well as those for smaller regions (Choi *et al.*, 1994) and should make it possible to amplify the 16S rRNA sequence from all bacteria (McDade and Anderson, 1996). The sequence obtained can be compared to available sequences (Genbank; EMBL) to provide an idea of closest relative (Busse *et al.*, 1996; McDade and Anderson, 1996). In addition to providing taxonomic information and classifying species (Tanner *et al.*, 1994), sequences suitable for oligonucleotide probes (Paster *et al.*, 1995) and PCR primers (McDade and Anderson, 1996) based on variable sequence regions (McDade and Anderson, 1996) can be obtained.

1.8.8.2 Application

The use of 16S rRNA sequence analysis for phylogenetic studies has been applied to the genus *Actinomyces* (Ramos *et al.*, 1997) as well as *Capnocytophaga*, *Treponema*, *Fusobacterium*, *Pasteurella*, *Selemonas* and *Veillonella* species (Paster *et al.*, 1995). It is accepted that both complete and partial rRNA gene sequences can be used for bacterial identification (Ludwig and Schleifer, 1994) but that partial rRNA sequences should not be used for phylogenetic groupings.

1.8.8.3 rRNA and taxonomy

Current phylogenetic analysis is based on comparative sequence analysis of rRNA. Ribonucleic acid (RNA) is a single stranded polymer composed of alternating units of the sugar ribose and a phosphate group, with nitrogen bases were uracil (U) replaces thymine (T) but still pairs with adenine (A). There are three types of RNA, all concerned with protein synthesis. Messenger RNA (mRNA) carries the genetic information concerning type and order of amino acids; transfer RNA (tRNA) carries the correct amino acids for protein synthesis to the ribosome and ribosomal RNA (rRNA) is a component of ribosomes where proteins are constructed. The section below will deal only with rRNA.

Prokaryotic rRNA is composed of three molecules; 5S, 16S and 23S. It makes a suitable target for studying genetic relatedness because by the nature of its function it is found in all organisms and will contain functionally conserved sequence regions as well as variable sequence regions which will exhibit variation between closely related organisms (Priest and Austin, 1993a). The rRNA adopts a secondary structure of stems and loops were the conserved regions form the stems and the variable regions the loops (Smith *et al.*, 1995). The 5S rRNA is composed of up to 116 for Gram-positive organisms to 120 nucleotides for Gram-negative organisms (Priest and Austin, 1993a) and its small size limits its usefulness. The 16S and 23S subunits are larger and therefore contain more genetic information than the 5S (Solignac *et al.*, 1991). The small 16S subunit has a length of approximately 1600 nucleotides, whilst the 23S is approximately 3,000 nucleotides (Olsen *et al.*, 1986). They are also present in a large copy number, up to 10,000 molecules of 16S rRNA are found per cell (Conrads and Brauner, 1993). Both 16S and 23S are composed of variable regions separated by conserved regions, the variable regions reflect the sequence of the genome (Stackebrandt and Liesack, 1993) and can therefore be used to distinguish species, whilst conserved sequences are species-specific. In addition, a functionally inert spacer region lies between the 16S and 23S genes which also has a variable sequence (Smith *et al.*, 1995). Realisation regarding the potential of this molecule means that there are accessible databases containing sequence information and sequence alignments to rRNA sequences within Genbank or EMBL allow phylogenetic comparisons (Göbel and Choi, 1995) at all taxonomic levels (Solignac *et al.*, 1991).

1.8.9 OTHER TECHNIQUES

In addition to those techniques mentioned in detail in section 1.8 above, many other techniques have been used for the identification, detection and studies of genetic heterogeneity of periodontal isolates. Restriction endonuclease analysis (REA) is the digestion of DNA with restriction endonucleases and subsequent separation of restriction fragments by gel electrophoresis. Restriction endonucleases are enzymes that cut DNA and RNA at specific recognition sites resulting in a number of fragments. The sizes of the fragments will depend on the position of the restriction sites in the DNA strand and when separated by gel electrophoresis (described in section 1.8.5.6), the smallest fragments will travel furthest. Different restriction patterns are due to different DNA sequences (Maslow *et al.*, 1993). The genetic diversity of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* in children of school age has been studied by this method (van

Steenbergen *et al.*, 1991). The use of multilocus enzyme electrophoresis (which is described in section 1.10.7.1) failed to find an association between the genotype of *P. gingivalis* and the disease type the isolate came from (Loos *et al.*, 1993) but will differentiate *Capnocytophaga* species (Frandsen *et al.*, 1996). Fluorescent tagged monoclonal antibodies (see section 1.10.6.1) have been used to detect periodontopathogens (*P. intermedia*, *P. gingivalis*, *A. actinomycetemcomitans* and *Fusobacterium nucleatum*) in plaque (Wolff *et al.*, 1992). Recently, monoclonal antibodies to *T. denticola*, pathogen-related oral spirochaetes (Riviere *et al.*, 1992) *T. socranskii* subsp. *buccale* and *T. socranskii* subsp. *socranskii* (Riviere and DeRouen, 1998) have enabled the identification and enumeration of spirochaete species in plaque and confirmed the association with disease and subgingival plaque (Riviere *et al.*, 1992).

1.9 *PREVOTELLA* SPECIES

1.9.1 TAXONOMY

Prevotella species are black-pigmenting, rod-shaped, non-spore forming obligately anaerobic organisms which until 1990 (Shah and Collins, 1990) were classified within the genus *Bacteroides*. Recently there have been many changes to the taxonomic status of this group, due to the recognition of the extensive heterogeneity in biochemical and chemical properties amongst the group. The genus *Bacteroides* is now restricted to include only bile-resistant species of the '*B. fragilis* group'; *B. caccae*, *B. distasonis*, *B. eggerthii*, *B. fragilis*, *B. merdae*, *B. ovatus*, *B. stercoris*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus* (Shah and Collins, 1989).

All asaccharolytic (non-carbohydrate fermenting) species were placed into a new genus (Shah and Collins, 1988), *Porphyromonas*. The latter included *B. gingivalis* (now *P. gingivalis*), *B. endodontalis* (*P. endodontalis*) and *B. asaccharolyticus* (*P. asaccharolytica*). Saccharolytic organisms with a predominantly oral habitat were assigned to the newly created genus *Prevotella* named after the French anaerobic microbiologist A. R. Prévot (Shah and Collins, 1990). These changes are summarised in table 1.4. This incorporated pigmenting, bile sensitive species within the '*B. melaninogenicus-B. oralis* group'; Member species include *B. intermedius* formerly *B. melaninogenicus* subspecies *intermedius* (now *P. intermedia*), *B. melaninogenicus* formerly *B. melaninogenicus* subspecies *melaninogenicus* (now *P. melaninogenica*), *B. loescheii* (*P. loescheii*) and *B. denticola* (*P. denticola*). Non-pigmenting, oral species within the '*B. oralis* group' including *B. bivius* and *B. disiens* which are phenotypically similar to *B. intermedius* (Shah,

1992) and can be isolated from the female genital tract (Shah, 1992) are also assigned to *Prevotella*. Two recent additions to the genus *Prevotella* are *P. tannerae* and *P. enoeca* (Moore *et al.*, 1994).

The taxonomic status of species proposed as members of *Bacteroides* including *B. gracilis, B. capillosus, B. coagulans, B. forsythus, B. tectum, B. ureolyticus*, (Shah, 1992; Shah and Collins, 1989), *B. levii* and *B. macacae* is unclear (Shah and Gharbia, 1992a), however many species previously classified as *Bacteroides* have now been given generic status, for example *B. succinogenes* now *Fibrobacter* (Montgomery *et al.*, 1988) and *B. praeacutus* now *Tissierella* (Collins and Shah, 1986). Sequencing of 16S rRNA has lead to the reclassification of *B. amylophilus* into a new genus *Ruminobacter* (Stackebrandt and Hippe, 1986) and has recently been shown to cluster the species *B. levii* and *B. macacae* with *Porphyromonas* species (cited by Paster *et al.*, 1994). This technique has also reclassified *Hallella seregens* and *Mitsuokella dentalis* as *Prevotella dentalis* (Willems and Collins, 1995).

Table 1.4 Changes in nomenclature of the saccharolytic organisms previously within the genus *Bacteroides* now conforming to the generic description *Prevotella* and new species recently assigned to this genus

	PREVIOUS TAXONOMIC STATUS	PRESENT TAXONOMIC STATUS	MOST RECENT CONSIDERATIONS
PIGMENTING ON BLOOD AGAR	*Bacteroides corporis*	*Prevotella corporis*	
	Bacteroides intermedius	*Prevotella intermedia*	*P. intermedia / P. nigrescens*
	Bacteroides loescheii	*Prevotella loescheii*	
	B. melaninogenicus	*P. melaninogenica*	
	-	-	*Prevotella tannerae*
	-	PINLOs	*Prevotella pallens*
NON-PIGMENTING ON BLOOD AGAR	*Bacteroides bivius*	*Prevotella bivia*	
	Bacteroides buccae	*Prevotella buccae*	
	Bacteroides buccalis	*Prevotella buccalis*	
	Bacteroides disiens	*Prevotella disiens*	
	B. heparinolyticus	*P. heparinolytia*	
	Bacteroides oralis	*Prevotella oralis*	
	Bacteroides oris	*Prevotella oris*	
	Bacteroides oulorum	*Prevotella oulorum*	
	B. ruminicola	*Prevotella ruminicola*	
	Bacteroides veroralis	*Prevotella veroralis*	
	B. zoogleoformans	*P. zoogleoformans*	
VARIABLE PIGMENT	*Bacteroides denticola*	*Prevotella denticola*	
	-	-	*Prevotella dentalis*
	-	-	*Prevotella enoeca*

Adapted from Marsh and Martin (1992).

FOOTNOTES TO TABLE 1.4:

1. *Prevotella heparinolytia* and *P. zoogleoformans* may be transferred back to *Bacteroides* (Jousimies-Somer, 1997).
2. Additional PINLOs may exist in addition to those conforming to the description of *P. pallens* (sections 1.11 and 4.18).

1.9.1.1 Heterogeneity within the species *Prevotella intermedia*

Heterogeneity within *P. intermedia* has been recognised for some years (Lambe, 1974). Antigenic studies of oral and non-oral isolates of the species *B. melaninogenicus* subspecies *intermedius* showed them to be antigenically distinct (Reed *et al.*, 1980) and van Steenbergen *et al.* (1982) demonstrated low DNA homology of the type strain ATCC 25611 (now *P. intermedia*) to other strains, thus extensive heterogeneity. Later DNA homology studies revealed two groups, one exhibiting homology to the type strain of *B. melaninogenicus* subsp. *intermedius* which was renamed *B. intermedius* and another designated *B. corporis* (Johnson and Holdeman, 1983). Heterogeneity within *B. intermedius* was demonstrated in immunological studies (Gmür and Guggenheim, 1983). Four monoclonal antibodies (MAbs) against *B. intermedius* were used (Gmür and Guggenheim, 1983) to distinguish three serotypes, which correlated to genotype (Gmür and Wyss, 1985). Serotype I corresponding to type strain ATCC 25611 now *P. intermedia* (Gmür and Guggenheim, 1983) previously *B. intermedius* genotype I (Gmür and Wyss, 1985), serotype II corresponding to ATCC 25261 now *P. nigrescens* (Gmür and Guggenheim, 1983) previously *B. intermedius* genotype II (Gmür and Wyss, 1985) and serotype III with homology to NCTC 9336 now *P. nigrescens* ATCC 33563 (Devine *et al.*, 1994) also previously *B. intermedius* genotype II (Gmür and Wyss, 1985). Three MAbs were later used to confirm (Dahlén *et al.*, 1990b) these groups with strains from serotype I being recognised by all MAbs, serotype II being recognised by the lack of reaction with *P. intermedia* specific (Devine *et al.*, 1994) MAb 40BI3.2.2 and serotype III by the weak reaction with only one MAb.

1.9.1.2 Taxonomy and reclassification of *P. intermedia*

The black pigmented organism *Bacteroides melaninogenicus* was first described in 1921 (Oliver and Wherry, 1921) and named *Bacterium melaninogenicum*. Three subspecies were described (Moore and Holdeman, 1973) *B. melaninogenicus* subspecies *assacharolyticus*, subspecies *melaninogenicus* and subspecies *intermedius*, which were subsequently elevated to species, level (Johnson and Holdeman, 1983). *B. intermedius* was later reclassified in the genus *Prevotella* as described in section 1.9.1 above and renamed *Prevotella intermedia* (Shah and Collins, 1990).

P. intermedia has been separated into two species on the basis of physiological tests. DNA-DNA hybridization with reference strains showed two distinct DNA homology groups amongst strains designated *P. intermedia* (Shah and Gharbia, 1992a). One group (those

strains comprising serotype I, see section 1.9.1.1 above) showed 72-92% hybridization with strain ATCC 25611 and less than 20% with strain ATCC 33563. The other group, which exhibited high levels of hybridization (81-98%) with strain ATCC 33563 and low levels with strain ATCC 25611 contained strains of serotype II and III which could not be differentiated. These were reclassified *Prevotella intermedia* (type strain ATCC 25611; serotype I) and *Prevotella nigrescens* (type strain ATCC 33563; serotype II and III) (Shah and Gharbia, 1992a).

1.9.2 MORPHOLOGY AND CHARACTERISTICS OF *PREVOTELLA* SPECIES

Pigmenting *Prevotella* species (see table 1.4) when grown on blood agar produce convex, circular, shiny, smooth colonies which exhibit dark brown-black pigmentation (*P. denticola* exhibits variable pigment production; Shah, 1992), and the colour develops 2-5 days after subculturing (Shah and Gharbia, 1992a). Non-pigmenting colonies on blood agar are translucent, opaque or grey (Shah and Collins, 1990). Weak haemolysis of the blood is seen. Two pigments are produced, protoporphyrin which is required for the synthesis of cytochrome b and protohaem which is required for the synthesis of respiratory quinones (cited by Marsh and Martin, 1992). It has been reported that there are other pigments yet to be identified (Shah and Gharbia, 1993a). Colonies examined under long-wave UV radiation (365nm) exhibit a red fluorescence due to protoporphyrin (Shah and Gharbia, 1992a). Growth is inhibited by 20% bile and haemin and menadione are required for growth (Shah and Collins, 1990). The DNA base composition of *Prevotella* species is within the range 40-52 mol% guanine plus cytosine (Shah and Collins, 1990) and 40-44 mol% for *P. intermedia* and *P. nigrescens* (Shah and Gharbia, 1992a). Major fermentation products from glucose metabolism are acetic and succinic acids (Shah and Collins, 1990). Members of the genus *Bacteroides* possess the enzymes of the hexose monophosphate shunt (pentose phosphate pathway); glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase which allow the anaerobic oxidisation of carbohydrates. *Prevotella* species lack these and instead they posses enzymes which allow catabolism via the Embden-Meyerhof pathway (Shah and Gharbia, 1993b) also known as glycolysis, the first step in anaerobic respiration (see section 1.8.4.1 for further details of bacterial metabolism).

1.9.2.1 Characteristics of *P. intermedia* and *P. nigrescens*

In addition to the properties demonstrated by *Prevotella* species (described in section 1.9.2 above), *P. intermedia* and *P. nigrescens* produce indole (Shah and Gharbia, 1992a). It is

worthy of note that *P. loescheii* and *P. zoogleoformans* can also give a positive result to a test for indole production (Shah and Collins, 1990). *P. intermedia* and *P. nigrescens* are also catalase negative (Fukushima *et al.*, 1992; Summanen *et al.*, 1993) and lipase positive (Shah and Gharbia, 1992a). Glucose fermentation by *Prevotella* species yields acetic and succinic acids, both *P. intermedia* and *P. nigrescens* also produce *iso*-butyric and *iso*-valeric acids (Shah and Gharbia, 1992a).

1.9.3 *P. INTERMEDIA* AND *P. NIGRESCENS* IN HEALTH AND DISEASE

Historical studies examining the relationship between microorganisms and periodontal health generally refer to *B. intermedius* without reference to genotype (I or II, now *P. intermedia* and *P. nigrescens*, Shah and Gharbia, 1992b) or serotype. The application of MAbs to samples from periodontal pockets detected a higher proportion of isolates of serotype I (*P. intermedia*) from this disease site (Dahlén *et al.*, 1990b). The association of *P. intermedia* with sites of active periodontal disease (Dzink *et al.*, 1985; Savitt and Socransky, 1984, Slots *et al.*, 1986; Zambon *et al.*, 1981) has been reported extensively, as well as minor disease sites and areas of health (Spiegel *et al.*, 1979; White and Mayrand, 1981; Zambon *et al.*, 1981). Since the taxonomic changes, *P. nigrescens* has been isolated from healthy sites in the oral cavity (Könönen, 1993), endodontic infections (Gharbia *et al.*, 1994), initial periodontitis (Mättö *et al.*, 1996b), oral abscesses (Milson *et al.*, 1996) and *P. intermedia* from deep periodontal pockets (Gharbia *et al.*, 1994; Milson *et al.*, 1996). However, more recent studies (Ashimoto *et al.*, 1996) detected high levels of both *P. intermedia* and *P. nigrescens* in periodontal pockets compared to gingivitis. *P. intermedia* (possibly incorporating unidentified *P. nigrescens*) has also been isolated from adults (Moore *et al.*, 1987) and children (Tsuruda *et al.*, 1995) with gingivitis, ANUG (Loesche *et al.*, 1982), periodontitis associated with systemic disease such as neutropenia and Papillon-Lefèvre Syndrome, as well as HIV-positive patients (Dahlén, 1993). Despite the taxonomic changes, *P. nigrescens* is not routinely tested for in all cases (Isoshima *et al.*, 1995).

The presence of *P. intermedia* and *P. nigrescens* has been positively correlated with the occurrence of the hormones testosterone, estradiol and progesterone at puberty (Nakagawa *et al.*, 1994) and during pregnancy (Kornman and Loesche, 1980).

It is now accepted that the two species differ in their site specificites (Devine *et al.*, 1994; Gharbia *et al.*, 1994), pathogenicity and role in periodontal disease (Devine *et al.*, 1994).

1.10 METHODS FOR IDENTIFICATION, DIFFERENTIATION AND STUDYING THE GENETIC HETEROGENEITY OF *P. INTERMEDIA* AND *P. NIGRESCENS*

As previously described (see section 1.9.1.1), the heterogeneity among strains of *P. intermedia*, lead to the division into two genetically distinct species, however their phenotypic similarities make routine speciation difficult. Many techniques have been used for species differentiation which is desirable to determine the distribution of the species within the oral cavity and studies investigating the clonal analysis of isolates will clarify the associations of each species with disease status and possible aetiological role (Mättö *et al.*, 1996b). The use of a particular characteristic for speciating bacteria is dependent on that particular characteristic being stable within strains of a species but variable between species (Maslow *et al.*, 1993). For strain typing, there must be variation between strains (Maslow *et al.*, 1993) but the result for a given strain must be highly reproducible.

1.10.1 SELECTIVE MEDIA AND CULTURE

Prevotella species can be successfully cultured on media supplemented with haem and vitamin K (Shah, 1992) and incubated in an anaerobic environment. Kanamycin-vancomycin laked blood agar selects for *Prevotella* and *Bacteroides* species (Summanen *et al.*, 1993). *P. intermedia* and *P. nigrescens* exhibit only weak haemolysis of blood agar (Shah and Gharbia, 1992a) and dark brown to black pigmented colonies can be selected for further study.

1.10.2 TRADITIONAL LABORATORY TESTS

All *Prevotella* species are small non-sporeforming, Gram-negative bacilli (Shah, 1992) displaying the characteristics described above in sections 1.9.2 (*Prevotella* species) and 1.9.2.1 (*P. intermedia* and *P. nigrescens* specifically). In all cases, the reactivity of *P. intermedia* and *P. nigrescens* is identical.

Conflicting information is available for lipase reaction. Initially, a lipase positive reaction was reported, with strains of *P. intermedia* showing a quicker reaction (after 24 h) on egg yolk agar than *P. nigrescens* (after 48 h) (Shah and Gharbia, 1992a). Later studies suggest the value of a lipase test as a differentiation tool is negligible due to the very weak positive results (Teanpaisan *et al.*, 1995) as well as negative results (Dahlén *et al.*, 1996). A positive lipase reaction is quoted in the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993).

1.10.3 (API) COMMERCIAL IDENTIFICATION KITS

Pure cultures may be tested by miniaturised commercial kits designed for the identification of anaerobic microorganisms (see section 1.8.3). The RapID ANA II system (Innovative Diagnostic Systems, Georgia) is designed 'for the biochemical identification of medically important anaerobic bacteria' and the Rapid ID 32 A kit (bioMérieux) is marketed as an 'identification system for anaerobes'.

Studies in the late 1980s point to the fact that the two recognised homology groups (corresponding to ATCC 25611 and NCTC 9336 or ATCC 25261) of *B. intermedius* (now known to indicate the two species) were not separated by the RapID ANA system (Dellinger and Moore, 1986) which was the forerunner to RapID ANA II system which is used in this study (section 2.10.1). The RapID ANA II system also fails to distinguish strains of *P. nigrescens* from *P. intermedia* as strains of both species will yield the same microcode and will be identified as *P. intermedia* (personal experience). The RapID ANA II differential chart for Gram-negative rods and the Rapid ID 32 A identification table as contained in the respective manufacturer's instruction booklets, lists those organisms identified by each system and includes only *P. intermedia.* This is also illustrated experimentally in tables 6.22 and 6.23. Therefore this method can be used to confirm a presumptive identification made by Gram stain and colony morphology but must be followed by further tests.

1.10.4 ELECTRON MICROSCOPY

Electron microscopy studies of the surface of strains of *B. intermedius* (Devine *et al.*, 1989) demonstrated fibrils on the surfaces of all strains examined, although the density, length and morphology was diverse. Thin-sectioning and negative-staining of strain ATCC 25611 (*P. intermedia*) highlighted four types of appendages, grouped according to diameter and occurrence, on the surface of *B. intermedius* (Leung *et al.*, 1989). A more recent study (Gharbia *et al.*, 1994) has shown differences in the surface properties of the reclassified species, demonstrating fimbria like projections only on the surface of *P. nigrescens* and a more pronounced capsular layer around *P. intermedia* (Gharbia *et al.*, 1994).

1.10.5 GAS-LIQUID CHROMATOGRAPHY

The theory of GLC is described in section 1.8.4.

Prevotella species; *P. corporis*, *P. denticola*, *P. intermedia*, *P. loescheii*, *P. melaninogenica* and *P. nigrescens* all produce acetic and succinic acids and all except *P.*

loescheii also produce *iso*-valeric and *iso*-butyric acids. *P. intermedia* and *P. nigrescens* also produce propionic acid. *P. denticola* and *P. melaninogenica* produce lactic acid and discrimination between *Porphyromonas* species occurs due to the production of butyric acid by members of this genus (Shah and Gharbia, 1992a). GLC is of little value for species (*P. intermedia* from *P. nigrescens*) differentiation using this technique. However, recent work on oral anaerobes by Takada and Hirasawa (1997) suggested that *P. intermedia* and *P. nigrescens* could be differentiated by the higher quantity of acetic acid produced by *P. nigrescens* compared to *P. intermedia*.

1.10.6 MONOCLONAL ANTIBODIES

1.10.6.1 Theory

The production of antibodies in response to bacterial antigens is exploited in the production of monoclonal antibodies (MAbs). MAbs are artificially produced from a single clone to a have a single specificity.

1.10.6.2 Application with reference to *P. intermedia* and *P. nigrescens*

Antigenic heterogeneity tested among strains of *B. intermedius* lead to the formation of three serogroups (Gmür and Guggenheim, 1983) as described in section 1.9.1.1. Three of the MAbs used by Gmür and Guggenheim (1983) were tested for their ability to differentiate strains of *P. intermedia* and *P. nigrescens* (Devine *et al.*, 1994). Of these, the specificity of one, MAb 40BI3.2.2 to strains of *P. intermedia* has been demonstrated (Devine *et al.*, 1994).

1.10.7 MULTILOCUS ENZYME ELECTROPHORESIS (MLEE)

1.10.7.1 Technique

The technique of MLEE is the study of enzymes in non-denaturing polyacrylamide electrophoresis gels and classifying strains by the presence or absence of bands and their electrophoretic mobilities (Priest and Austin, 1993b). The mobility of an enzyme is related to the amino acid sequence of the gene and will therefore detect genetic differences.

1.10.7.2 Application with reference to *P. intermedia* and *P. nigrescens*

All members of the genus *Prevotella* possess the enzymes glutamate dehydrogenase and malate dehydrogenase (Shah and Collins, 1990). Electrophoresis of cell free extracts provides a mobility pattern for these enzymes and the speed at which the enzymes migrate

allows the species to be separated. Enzymes from those strains exhibiting DNA homology to ATCC 25611 (*P. intermedia*) migrate faster (Shah and Gharbia, 1992a, b; Frandsen *et al.*, 1995). than those of *P. nigrescens* (Shah and Gharbia, 1992a, b). The slow migrating group included strains of both serotype II and III (Shah and Gharbia,1992b).

MLEE is often used to classify isolates as *P. intermedia* or *P. nigrescens* before or after they are tested by other techniques, such as REA (Frandsen *et al.*, 1995) and oligonucleotide probes (Mättö *et al.*, 1996b) as confirmation of classification.

1.10.8 PROTEIN ELECTROPHORESIS

1.10.8.1 Technique

Whole cell proteins from cultivatable species can be examined by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions in the presence of the anionic detergent SDS; sodium dodecyl sulphate (Kersters *et al.*, 1994). Proteins are separated by weight and visual comparison readily highlights similarities and differences (Kersters *et al.*, 1994) differentiating at the species level.

1.10.8.2 Application with reference to *P. intermedia* and *P. nigrescens*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used to study whole cell proteins and outer membrane material (Cookson *et al.*, 1996) of *P. intermedia* and *P. nigrescens*. Many potential species-specific markers have been suggested. Gharbia *et al.* (1994) demonstrated a 31 kDa protein band unique to *P. nigrescens* which was not detected in other studies (Cookson *et al.*, 1996; Dahlén *et al.*, 1996). A later study suggested that both *P. intermedia* and *P. nigrescens* displayed a band at 29 kDa, but that differentiation of *P. intermedia* was possible by an additional 25 kDa band (Dahlén *et al.*, 1996). Cookson *et al.* (1996) demonstrated a 21 kDa band identifying *P. intermedia* strains and two bands of 18 kDa and 22 kDa identifying *P. nigrescens*. Work by Teanpaisan *et al.* (1995) highlights the lack of evidence for marker bands seen between studies, reporting that certain bands could differentiate strains of either species but that they were many and variable. All strains of *P. intermedia* displayed a band at 18.7 kDa and some displayed one at 32 kDa. All *P. nigrescens* strains had bands of 32 kDa, which could be equivalent to the band reported by Gharbia *et al.* (1994), and one at 17.2 kDa as well as a protein band of either 19.6 kDa or 21 kDa. These three bands (17.2 kDa, 19.6 kDa and 21 kDa) are all within a similar range to those reported by Cookson *et al.* (1996). A study by Milson *et al.* (1996) reported higher molecular weight bands, 4 in the range 31 kDa-100

kDa in *P. nigrescens* strains and 2 of 44 kDa and 58 kDa in *P. intermedia*. The inconsistencies may reflect minor differences in the electrophoretic techniques used, protein extraction procedure or interpretation of the band sizes when compared to different protein ladders. Alternatively, variation in bacterial culture conditions is known to be responsible for variable surface protein expression (Dahlén *et al.*, 1996). All reported studies confirm that this technique will differentiate the two species, however, culture conditions and electrophoretic techniques must be standardised to allow inter-laboratory comparisons.

1.10.9 DNA-DNA HYBRIDIZATION

1.10.9.1 Technique

The technique of DNA-DNA hybridization exploits the ability of single stranded DNA to reassociate to become double stranded DNA under suitable conditions (Stackebrandt and Liesack, 1993). It allows an indication of sequence homology between two species to be obtained based on the entire genome, providing a reflection of genetic divergence (Johnson, 1991). Values of sequence similarities are provided by the extent to which DNA from one organism hybridises with DNA from another (Johnson, 1991) and a value over 70% is considered a species relationship (Wayne *et al.*, 1987). Controlled conditions are required especially regarding temperature which affects the reaction rate and specificity and salt concentration which is essential for hybridization (Johnson, 1991).

1.10.9.2 Application with reference to *P. intermedia* and *P. nigrescens*

This technique was instrumental in the division of *P. intermedia* into two species (Shah and Gharbia, 1992a) as described in section 1.9.1.2 above and has been used to speciate clinical isolates identified as *P. intermedia* (Fukushima *et al.*, 1992).

1.10.10 PCR

The theory of the PCR is described in section 1.8.5.1.

An increase in knowledge regarding the 16S rRNA gene sequence has allowed it to become a target for PCR primers (Ashimoto *et al.*, 1996; Conrads *et al.*, 1997) which are species-specific. The success of *P. intermedia* or *P. nigrescens* specific primers has been confirmed by MLEE (Conrads *et al.*, 1996).

1.10.11 RAPD-PCR

The theory of RAPD-PCR is described in section 1.8.6.1.

RAPD-PCR analysis of clinical isolates identified as either *P. intermedia* or *P. nigrescens* has been performed using random primer OPA-13 (Mättö *et al.*, 1996a) in an attempt to speciate them. A 900-bp product was detected in all *P. intermedia* isolates except one and a 1.3-kb product was detected in all isolates originally identified as *P. nigrescens* except 2 (Mättö *et al.*, 1996a). Clonal analysis with random primer OPA-13 yielded 4 distinct amplification profiles for 37 isolates of *P. intermedia* and 6 for 91 isolates of *P. nigrescens* (Mättö *et al.*, 1996a). Random primer OPA-03 demonstrated greater heterogeneity with 5 profiles for 37 *P. intermedia* isolates and 17 profiles for 91 isolates of *P. nigrescens* (Mättö *et al.*, 1996a).

1.10.12 NUCLEIC ACID PROBES

The theory is described in section 1.8.7.1.

Dix *et al.* (1990) described 8 oligonucleotide probes based on 16S rRNA sequences, 5 (1Bi-1, 2, 3, 5, 6) for the identification of *B. intermedius* I (*P. intermedia*), 2 (2Bi-1, 2) for *B. intermedia* II (*P. nigrescens*) and 1 (Bi-4) designed to both types I and II which were specific when tested against other bacterial species. The potential of these sequences has since been exploited in other studies. A high specificity of probe 1Bi-4 has been reported (Conrads and Brauner, 1995). The speciation of clinical isolates using 1Bi-1 (*P. intermedia*) and 2Bi-1 (*P. nigrescens*) positioned isolates in the same species groups as MLEE (Mättö *et al.*, 1996b). Some cross-reactivity between *P. intermedia* and *P. nigrescens* probes was seen but identifications were made on the strength of signal (Mättö *et al.*, 1996b) and there was no reactivity with other species.

Seven oligonucleotides, excluding Bi-4 because it is undesirable to detect both species, were analysed for their ability to hybridise strains of *P. intermedia* or *P. nigrescens* (Shah *et al.*, 1995). Only 1Bi-1 and 2Bi-1 were considered suitably placed within the 16S sequence to be tested and under high stringency conditions both were specific (Shah *et al.*, 1995). The typing of clinical isolates with these probes correlated with the identifications obtained by MLEE and DNA-DNA hybridization (Shah *et al.*, 1995). An oligonucleotide sequence (BI) described by Chuba *et al.* (1988) for the detection of *P. nigrescens* has demonstrated the ability to hybridise with both *P. intermedia* and *P. nigrescens* (Shah *et al.*, 1995).

1.10.13 RIBOSOMAL RNA GENE SEQUENCING - 16S RRNA

The theory of Ribosomal RNA gene sequencing is described in section 1.8.8.1 and the value for taxonomic analysis is outlined in section 1.8.8.3.

Paster *et al.* (1994) sequenced 95% of the 16S rRNA gene of black-pigmenting microorganisms including *P. intermedia, P. nigrescens, P. melaninogenica, P. gingivalis* and *P. endodontalis*; non-pigmenting *Prevotella* species including *P. zoogleoformans* and *P. heparionlytica* as well as other *Bacteroides* species. A 94.7% similarity was demonstrated between *P. intermedia* and *P. nigrescens*, sufficient to warrant separate species distinction and to provide a suitable method for differentiation of the two species. These sequences can be found in the EMBL database (for details see appendix 6.6, table 6.3) and were used for work described in materials and methods section 2.6.8 and 2.7.

1.10.14 RESTRICTION ENDONUCLEASE ANALYSIS (REA), RIBOTYPING AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

1.10.14.1 Technique

Restriction enzyme analysis (described in section 1.8.9) and ribotyping (hybridization of rRNA with digested DNA; Milson *et al.*, 1996) use restriction endonucleases to cleave DNA at its specific sites and to study the pattern of DNA fragments produced. RFLP is, in other words, the detection of small changes (or differences) in the DNA nucleotide sequence. The RFLP will become apparent when the DNA is cut with a restriction endonuclease and the pattern of fragments (restriction map) analysed.

1.10.14.2 Application

The digestion of genomic DNA/rRNA with restriction enzymes has been used to characterise strains of *P. intermedia* and *P. nigrescens* (Dahlén *et al.*, 1996; Devine *et al.*, 1997; Frandsen *et al.*, 1995; Pearce *et al.*, 1996; van Steenbergen *et al.*, 1991). The enzymes *Eco*R1 (Frandsen *et al.*, 1995) and *Taq* I (Devine *et al.*, 1997) demonstrated REA and ribotype patterns respectively, that differentiated the species and were distinct to either *P. intermedia* or *P. nigrescens*. Frandsen *et al.* (1995) reported considerable intra-species heterogeneity for both species and it has been suggested that it is greater among strains of *P. nigrescens* (Devine *et al.*, 1997). The use of *Pst* I provided two distinct patterns which corresponded to the species (Dahlén *et al.*, 1996). Pattern A with no fragments below 3.9 kb was seen for *P. intermedia*, pattern B showed fragments below 2.8 kb for *P. nigrescens*. The same enzyme demonstrated 8 unique REA digest patterns among strains of *P. intermedia* isolated from school children with periodontitis (van Steenbergen *et al.*, 1991), although no reference was made to *Prevotella nigrescens* or serotype of *P. intermedia*. Characterised strains and clinical isolates of *P. intermedia* and *P. nigrescens* were analysed

by digestion of genomic DNA with enzyme *Msp*-I which delineated two groups identical to those shown by MLEE (Frandsen *et al.*, 1995) i.e. one corresponding to *P. intermedia* and one to *P. nigrescens*. In another study, REA failed to differentiate the two species (Pearce *et al.*, 1996).

This technique has also been applied to PCR amplified 16S rRNA genes (PCR-RFLP) from both species (Milson *et al.*, 1996). Three restriction enzymes were shown to produce species-specific profiles which corresponded to groupings made by other techniques (Milson *et al.*, 1996).

Ribotyping of clinical isolates has suggested transmission of *P. intermedia* and *P. nigrescens* between spouses (Mättö *et al.*, 1996b).

All techniques used validate the differentiation of *P. intermedia* into two separate species, and are of value for the typing of clinical isolates. Ribotyping, REA and RAPD-PCR in particular, demonstrate considerable intra and inter-species heterogeneity indicating the need for further taxonomic studies.

1.11 *PREVOTELLA INTERMEDIA* AND *PREVOTELLA NIGRESCENS* LIKE ORGANISMS (PINLOS)

PINLOs were first described by Könönen in 1996 (cited by Jousimies-Somer, 1997) as Gram-negative rods which are biochemically similar to *P. intermedia* and *P. nigrescens* (Könönen *et al.*, 1998b). Unlike *P. intermedia* and *P. nigrescens*, they are faintly pigmenting (beige to brown) and lipase negative. These are two features which will routinely differentiate PINLOs from either *P. intermedia* or *P. nigrescens*. PINLOs have different AP-PCR (RAPD-PCR) profiles than *P. intermedia* and *P. nigrescens* and are not detected by *P. intermedia* or *P. nigrescens* specific probes (Könönen *et al.*, 1998b). Complete 16S rRNA sequence information confirmed the assignment of PINLOs to the genus *Prevotella*, displaying most similarity to *P. intermedia* and *P. nigrescens* but differing from all previously described species (Könönen *et al.*, 1998b). This finding resulted in the classification of a new species *Prevotella pallens* (Könönen *et al.*, 1998a).

PINLOs (*P pallens*) have been isolated from odontogenic abscesses, perimplantitis (Finegold and Jousimies-Somer, 1997) and other oral samples including a patient with osteomyelitis of the mandible (Könönen *et al.*, 1998a).

Other PINLOs exist in addition to those renamed as *P. pallens*, three non-oral isolates phenotypically identical to and biochemically (GLC) identified as *P. intermedia* have been

examined in relation to *P. intermedia* and *P. nigrescens* (Devine *et al.*, 1994 and subsequently during this study (section 2.13).

1.12 AIMS OF STUDY

Since the reclassification of *P. intermedia* into two species, many attempts have been made to find a successful, rapid and reproducible technique to differentiate isolates of the two species. *P. intermedia* and *P. nigrescens* are putative periodontopathogens and their identification is desirable to determine the species associations with disease in the oral cavity. The presence of these species within the oral cavity of patients with Papillon-Lefèvre Syndrome strengthens the association with periodontal disease.

The use of cultivation based techniques to identify *P. intermedia* and *P. nigrescens* from clinical microbiological samples is ineffective and therefore molecular based techniques (RAPD-PCR, PCR and partial 16S rRNA gene sequencing) were tested for suitability in our hands. The success of these techniques to perform this task was evaluated using previously characterised strains of both species. The potential of RAPD-PCR as a molecular differentiation tool was also assessed using recently obtained clinical isolates identified only as *P. intermedia* and PINLO strains thought to be related *to P. intermedia* and *P. nigrescens*. The success of RAPD-PCR is compared to the established technique of partial 16S rRNA gene sequencing. In addition, 16S rRNA gene sequence information was analysed and used to design potentially species-specific PCR primers. These were tested in parallel with previously published PCR primers against known strains of *P. intermedia* and *P. nigrescens* and clinical isolates, for their ability to provide reproducible identification of either species.

A comprehensive study of the microorganisms isolated from the oral cavities of patients with PLS was undertaken. Microorganisms included *P. intermedia* and *P. nigrescens* which were examined in detail along with culture collection strains. Genus level identifications were undertaken using commercial identification kits, traditional laboratory techniques and Gas-Liquid Chromatography. A comparison of the identification obtained by these methods and that obtained by partial 16S rRNA gene sequencing was undertaken.

Summarised, the aims of this study were to optimise the techniques of;

- RAPD-PCR
- PCR using species-specific primers
- Partial 16S rRNA gene sequencing

These techniques were used to test the following hypotheses;

- That RAPD-PCR can differentiate known strains of *P. intermedia* from strains of *P. nigrescens* and be applicable to grouping unknown *Prevotella* species.
- That species-specific PCR primers based on 16S rRNA gene sequence information can be designed and used for the differentiation and identification of *P. intermedia* and *P. nigrescens*.
- That RAPD-PCR, PCR with species-specific primers and partial 16S rRNA gene sequencing are all capable in our hands of differentiating *P. intermedia* and *P. nigrescens*, including isolates identified only as *P. intermedia* (using commercial anaerobe identification kits).
- That RAPD-PCR and partial 16S rRNA sequencing can usefully permit comparison of PINLO strains with *P. intermedia* and *P. nigrescens*.
- That the use of partial 16S rRNA gene sequencing in addition to commercial identification kits (API), traditional laboratory techniques and GLC can improve identification of microorganisms isolated from the gingival pocket of patients with Papillon-Lefèvre Syndrome.

2. MATERIALS AND METHODS

2.1 MATERIALS LIST

Details of all chemicals and compounds used in this study are listed below.

Advanced Biotechnologies, Epsom, Surrey

Thermostable (*Taq*) DNA polymerase (250 units) supplied with vials of $MgCl_2$ and buffer IV

API - see bioMérieux

Appligene, Birtle, County Durham

Agarose

BDH Merck Ltd, Merck House, Poole, Dorset

Acetic acid (glacial; Analar)

Acetone (Analar)

Ammonium acetate

Amyl Alcohol

Bromophenol blue

Calcium chloride (fused granular 4-20 mesh)

Chloroform (Analar)

Diethyl ether (Analar)

Ethanol (Analar)

Glucose (Analar)

Glycerol (Analar)

Hydrogen peroxide (Analar)

Iso-amyl alcohol (Analar)

Lactic acid (mixture of D-lactic and L-lactic; Analar)

Methanol (Analar)

Oil of cedarwood (thickened for oil immersion)

Ortho-phosphoric acid

Propan-2-ol (Analar)

Sodium chloride (Analar)

Sodium dodecyl sulphate

Sucrose

Succinic acid (Analar; 99.5% purity)

Sulphuric acid (Analar)

Tetramethyl - p- phenylenediamine

Becton Dickinson (UK) Ltd, Oxford

Peptone

bioMérieux UK Ltd, Basingstoke, Hampshire

Commercial identification kits; API 20 NE, Rapid ID 32 Strep, Rapid ID 32 A, API Coryne, API NH

Mineral oil (for identification kits)

Test reagents; James, FB, VPA,VPB, NIT 1, NIT 2, ZYM A, ZYM B, PYZ, NIN

BOC - Special gases

Anaerobic Gas Mixture (10% hydrogen, 10% carbon dioxide and 80% nitrogen)

BOC - Industrial gases, Worsley

High purity hydrogen

Compressed air

Nitrogen (oxygen free)

Boehringer Mannheim, Mannheim, Germany

Proteinase K

Fisher Scientific UK, Loughborough, Leicestershire

Phenol dissolved in Tris

Flowgen Instruments, (Gentra Systems Inc.) Lynn Lane, Shenstone, Staffordshire

Puregene DNA Isolation Kit

Gentra Systems Inc. - see Flowgen Instruments

Innovative Diagnostic Systems - See Pro-Lab Diagnostics

Lab M Ltd, Bury, Lancashire

Agar Number 2

Brain Heart Infusion Broth

Columbia Agar Base

Egg Yolk emulsion

Fastidious Anaerobe Agar

Fastidious Anaerobe Broth

GLC broth

Tryptone Yeast Cysteine agar

Yeast Extract

Metlab Supplies, Hawarden, Flintshire

Chart recorder paper (Kipp & Zonen BD8 multirange recorder)

Chart recorder pens (Kipp & Zonen BD8 multirange recorder)

New England Biolabs (UK) Ltd, Hitchin, Hertfordshire

ϕX174 DNA *Hae* III digest

Oligonucleotide Synthesising Service, School of Biological Sciences, University of Manchester

Primers; L10, RSP, US, 970-11, RE-TPU1, RE-RTU3, 1Bi-1, 2Bi-1

Oswals DNA Service, University of Southampton

Sequencing reactions and data

Oxoid (Unipath) Ltd, Basingstoke, Hampshire

Defibrinated horse blood

Perkin Elmer Applied Biosystems UK, Warrington, Cheshire

ABI PRISM™ Terminator Cycle Sequence Ready Reaction Kit

ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit

Primers; L10, RE-TPU1

Pharmacia Biotech Ltd, St. Albans, Hertfordshire

DNA polymerisation mix (10 μmol each dATP, dCTP, dGTP, dTTP. Concentration 20mM)

Polaroid, St. Albans, Hertfordshire

Photographic film (667 and 665)

Pro-Lab Diagnostics, Merseyside

RapID Ana II system

Qiagen, Crawley, West Sussex

QIAquick PCR Purification Kit

Taq DNA polymerase (250 units) supplied with vials of $MgCl_2$ and PCR buffer

Sigma Chemical Company Ltd, Poole, Dorset

Acetic acid

Ammonium sulphate

Bovine albumin

Bromothymol blue

Iso-butyric acid

n-Butyric acid (capillary GC, 99% purity)

Cysteine hydrochloride

Dimethyl-α-naphthylamine

EDTA

Haemin (equine)

Lambda *Pst* I digest

Lysosyme

Malonic acid (propanedioic acid; 99%)

Menadione (Vitamin K_3: 2-methyl-1,4-naphthoquinone)

Mineral oil (for PCR reactions)

Oxalic acid (ethanedioic acid; 99% purity)

Potassium nitrate

Potassium phosphate monobasic (99% purity)

Potassium phosphate dibasic trihydrate (99% purity

Sodium carbonate

Sulphanilic acid

Trizma base

Uncut λ DNA

Iso-valeric acid

n-Valeric acid (pentanoic acid; 99% purity)

TAAB Laboratories equipment Ltd, Calleva Park, Aldermaston, Berkshire

Carbol fuchsin (Gram) concentrate

Crystal violet (Gram) concentrate

Malachite green (Zn) concentrate

Lugol's iodine (Gram) concentrate

Safranin (Gram) concentrate

TCS Biologicals Ltd, Claydon, Buckinghamshire

Sterile product donor horse serum

Unipath Ltd - see Oxoid

2.2 BUFFERS, DYES AND SOLUTIONS

Below is a list of components of all buffers, dyes and other solutions used during this research.

Please note the following symbols:

† Accurate concentrations not supplied by manufacturer.

‡ Exact components not supplied by manufacturer.

Buffer IV 10x (Advanced Biotechnologies)	200 mM $(NH_4)_2SO_4$
	750 mM Tris HCl
	0.1% w/v Tween

Buffers EB, PB and PE (QIAquick PCR Purification Kit)‡

Cell lysis solution (Puregene DNA isolation kit)‡

Cell suspension solution (Puregene DNA isolation kit)‡

DNA hydration solution (Puregene DNA isolation kit)‡

Glycerol loading dyes (agarose gel electrophoresis)	Bromophenol blue (1 mg/ml)
	Xylene cyanol (1 mg/ml)
	Glycerol 30% v/v
	500 mM EDTA (pH 8.0)
	1M Tris (pH 7.0)
	dH_2O to 10 ml (final volume)

Lytic enzyme solution (Puregene DNA isolation kit)‡

Molecular weight markers	Lambda *Pst* I digest16.66% v/v
	Dyes (as above)16.66% v/v
	Buffer IV 8.3% v/v
	dH_2O to 12 ml (final volume)

PCR Buffer 10x (pH 8.7; Qiagen)	Tris-HCl[†]
	KCl[†]
	$(NH_4)_2SO_4$[†]
	15 mM $MgCl_2$

Protein precipitation solution (Puregene DNA isolation kit)[‡]

RNase A solution (Puregene DNA isolation kit)[‡]

STE (pH 8.0)	25% w/v sucrose
	50 mM Tris-chloride
	50 mM EDTA
	dH_2O to 100 ml (final volume)
TE (pH 8.0)	10 mM Tris-chloride
	1 mM EDTA
	dH_2O to 100 ml (final volume)
TPE (10x; pH 8.0)	Trizma base 1.08% w/v
	85% HPO_4 1.55v/v
	20 mM EDTA
	dH_2O to 1000 ml (final volume)

2.3 MICROBIOLOGY

2.3.1 Microorganisms

The strains and their sources used in this study are in table 2.1.

2.3.2 Growth and maintenance of anaerobic bacterial species

The preparation and sterilisation of the medium detailed below is described in appendix 6.1.

2.3.2.1 Solid medium

Prevotella species, including *P. corporis* and *P. pallens*, PINLO strains, *P. gingivalis*, *C. ochracea*, and facultative anaerobes from clinical sources were grown on Fastidious Anaerobe Agar (FAA) supplemented with 5% defibrinated horse blood. They were grown in an atmosphere of hydrogen, carbon dioxide and nitrogen (10:10:80% v/v) in an anaerobic cabinet (Compact M, Don Whitley Scientific, Shipley, W. Yorkshire) at 37°C until pigment developed. Prior to testing with a commercial identification kit, isolates were grown on Columbia Agar Base supplemented with 5% defibrinated horse blood (CBA) at 37°C in an anaerobic environment. *P. intermedia*, *P. corporis* and *P. pallens* strains tested for lipase activity were grown on egg yolk agar in an anaerobic environment.

Preparation and composition of all solid media is described in appendix 6.1.3.

2.3.2.2 Liquid medium

Liquid medium was inoculated with a 72 h colony taken from a FAA plate and maintained in an anaerobic environment as described above. Microorganisms were grown in Fastidious Anaerobe Broth (FAB) for 24 h before DNA extraction, Brain Heart Infusion Broth (BHI) for 3 days prior to testing Gram reaction and morphology and in Gas Liquid Chromatography Broth (GLC broth) for 3 days before harvesting fermentation products for analysis (described in methods section 2.11.3). Preparation and composition of all liquid media is described in appendix 6.1.4.

Table 2.1 Bacterial strains and their sources

BACTERIAL STRAIN	SUPPLIED BY
Prevotella intermedia ATCC 25611	Dr. D. Drucker*
Prevotella intermedia MH3	Dr. D. Drucker*
Prevotella intermedia MH6	Dr. D. Drucker*
Prevotella intermedia MH12	Dr. D. Drucker*
Prevotella intermedia MH15	Dr. D. Drucker*
Prevotella nigrescens ATCC 25261	Dr. D. Drucker*
Prevotella nigrescens ATCC 33536	Dr. D. Drucker*
Prevotella nigrescens MH1	Dr. D. Drucker*
Prevotella nigrescens MH2	Dr. D. Drucker*
Prevotella nigrescens MH4	Dr. D. Drucker*
Prevotella nigrescens MH5	Dr. D. Drucker*
Prevotella nigrescens LM94	Dr. D. Drucker*
PINLO A391	Dr. D. Devine
PINLO HST 1156	Dr. D. Devine
PINLO HST 2160	Dr. D. Devine
Prevotella pallens NCTC 130Y2	Dr. E. Könönen
Prevotella corporis ATCC 33547	Dr. D. Devine
Prevotella corporis A353	Dr D. Devine
Prevotella corporis A350	Dr. D. Devine
Porphyromonas gingivalis ATCC 33227	Dr. D. Love
Actinobacillus actinomycetemcomitans ATCC 29525	Prof. W. MacFarlane
Capnocytophaga ochracea W42	Prof. W. Wade
Staphylococcus aureus	Dr. N. M. Sayers

Dr. D. Devine, Oral Microbiology, Leeds Dental Institute, Leeds, UK.

Dr. D. Drucker, Biological Sciences, University of Manchester, UK

Dr. D. Love, University of Sydney, Sydney, Australia.

Dr. E. Könönen, Department of Bacteriology, National Health Institute, Helsinki, Finland.

Dr. N. M. Sayers, Biological Sciences, University of Manchester, UK

Prof. W. MacFarlane, Glasgow Dental School, Glasgow, Scotland.

Prof. W. Wade, Guys Hospital, London, UK.

* Originally isolated by M. Haapasalo, University of Helsinki, Finland.

2.3.3 GROWTH AND MAINTENANCE OF AEROBIC BACTERIAL SPECIES

The preparation of the medium detailed below is described in appendix 6.1

2.3.3.1 Solid medium

Aerobic clinical isolates were maintained on CBA in an aerobic environment at 37°C. Isolates were grown on CBA for 24 h before using an identification kit.

Tryptone Yeast Cysteine Agar (TYC) was used to grow suspected streptococcal species to test for extracellular polysaccharide production. Preparation and composition of all solid media is described in appendix 6.1.3.

2.3.3.2 Liquid medium

Isolates were grown in BHI for 24 h at 37°C in an aerobic environment prior to DNA extraction or Gram staining. Preparation and composition of all liquid media is described in appendix 6.1.4.

2.3.4 Long-term storage of microorganisms in FUM

Forty-eight hour colonies were transferred to a long term storage fluid medium (FUM) first described by Loesche *et al.* (1972) and modified by Gmür and Guggenheim (1983) and for storage at -80°C. The preparation and composition of FUM is described in appendix 6.1.5. FUM was pre-reduced for 24 h in an aerobic environment or an atmosphere of 10% hydrogen, 10% carbon dioxide and 80% nitrogen in an anaerobic cabinet (Compact M, Don Whitley Scientific) at 37°C. FUM was inoculated with colonies from a 48 h plate culture and incubated for 24 h at 37°C aerobically or in the anaerobic cabinet. After 24 h 5% (v/v) sterile glycerol was added and mixed before FUM was aliquoted into 1.5 ml sterile eppendorfs for storage at -80°C.

MATERIALS AND METHODS

SECTION A: MOLECULAR (DNA) TECHNIQUES

2.4 DNA EXTRACTION

2.4.1 GRAM-NEGATIVE BACTERIA

The technique described below was used to extract DNA from all strains of *P. intermedia*, *P. nigrescens*, PINLO, other putative periodontopathogens (listed in table 2.1 above) and all Gram-negative clinical isolates.

Each bacterial culture in FAB (5 ml) was centrifuged at 3,000 rpm (800 g) for 10 min and the cells resuspended in STE (0.5 ml). Lysosyme (20 µl) at a final concentration of 2 mg/ml was added before incubating at 37°C for 30 min. The addition of 7.5 µl proteinase K (final concentration 0.3 mg/ml) and 60 µl sodium dodecyl sulphate SDS, (final concentration 1%) was followed by incubation at 55°C for one hour to induce cell lysis. Phenol/chloroform/*iso*-amyl alcohol (25:24:1, v/v/v) was added (600 µl) and each suspension was mixed thoroughly on a vortex mixer. Following centrifugation at 13,000 rpm (16,060 g) for 10 min, 24 µl NaCl and 600 µl propan-2-ol were added to the nucleic acid containing supernatant fluid. Gentle mixing and incubation at 0°C for 10 min was followed by centrifugation at 13,000 rpm for 10 min. The pellet was resuspended in water and 100 µl ammonium acetate (final concentration 2.5M) was added. The suspension was incubated for one hour at 0°C before centrifugation (13,000 rpm for 20 min). Ice-cold ethanol (600 µl; stored at -85°C) was added to the supernatant to precipitate the DNA. After centrifugation (13,000 rpm, 10 min), the DNA was washed in 500 µl 70% ethanol (stored at -85°C). After removal of the ethanol, the pellet was resuspended in 100 µl TE overnight at 4°C. Extracted DNA was stored at 4°C.

2.4.1.1 RNA removal

DNA was extracted detailed in section 2.4.1 with an additional step. Before addition of 100µl ammonium acetate (final concentration 2.5 M), 2 µl of RNase A (10 mg/ml; Smith *et al.*, 1989b) was added and incubated for 1 h at 50°C (Smith *et al.*, 1989b). The extraction was completed as described in section 2.4.1.

2.4.2 GRAM-POSITIVE BACTERIA

DNA was extracted from all Gram-positive clinical isolates using Puregene DNA isolation kit (Gentra Systems, Inc.), according to the manufacturer's instructions (for details see appendix 6.2.1).

2.4.3 QUANTIFICATION OF AMOUNT OF DNA

Concentrations of DNA in 5 µl of each extraction were visually compared with a known amount of uncut λ DNA (400 ng/µl, 200 ng/µl, 100 ng/µl, 40 ng/µl) by electrophoresis of 10 µl in 0.8% agarose gels. Gels were stained in ethidium bromide (1 µg/ml in 1 x TPE), visualised under ultraviolet light (λ = 254 nm) and photographed on Polaroid 667 film. The DNA preparations were diluted in water where appropriate to obtain approximately equal concentrations for each sample.

2.5 RAPD-PCR

DNA (5 µl) was subjected to PCR using a Crocodile II™ thermal cycler (Appligene, County Durham). The reaction mix had a total volume of 50 µl which consisted of *Taq* polymerase (2.5 units), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) and 1 x buffer IV. The concentrations of $MgCl_2$ and random primer used respectively were 2.0 µM and 3.0 mM (for primer L10), 1.5 µM and 3.0 mM (for primer RSP), 1.0 µM and 2.0 mM (for primer US) and 1.0 µM and 3.0 mM (for primer 970-11). The random primers are described in table 2.2. The reaction mix was covered by an equal volume (50 µl) of mineral oil to prevent evaporation before subjecting to PCR.

The thermal cycling profiles were as follows;

For primer 970-11;
1 cycle of 4 min at 94°C, 1 min at 32°C, 1 min at 72°C
29 cycles of 1 min at 94°C, 1 min at 32°C, 1 min at 72°C
5 cycles of 1 min at 94°C, 1 min at 32°C, 5 min at 72°C.

For primers RSP, L10, US;
1 cycle of 4 min at 94°C, 1 min at 40°C, 1 min at 72°C
4 cycles of 1 min at 94°C, 1 min at 40°C, 1 min at 72°C
24 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C
1 cycle of 1 min at 94°C, 1 min at 55°C and 7 min at 72°C.

2.5.1 NEGATIVE CONTROLS

Negative controls were included in all RAPD-PCR reactions, consisting of all components of reaction mix other than template DNA. Sterile water was added in place of template.

2.5.2 OPTIMISATION OF RAPD-PCR CONDITIONS

Optimal concentrations of $MgCl_2$ and primer were determined using a titration series. RAPD-PCR was carried out as previously described in section 2.6, using $MgCl_2$

concentrations in the range 0.5 mM - 5 mM and primer concentrations in the range 0.5 μM - 5 μM.

2.5.3 ELECTROPHORESIS OF PCR PRODUCTS

PCR products (10 μl) were separated in 1.8% agarose gels at 110 V for 4 h, stained with ethidium bromide and visualised under ultraviolet light (λ = 254 nm). A λ *Pst* I digest (270 ng/μl) was used a size marker. Gels were photographed on Polaroid 665 and 667 films and band patterns compared visually.

2.5.4 REPRODUCIBILITY OF RESULTS

All RAPD analyses were performed on at least three separate occasions to check day-to-day reproducibility and 2 reactions were performed within each PCR to check inter-experimental variation. At least 2 separate extractions for each species tested were used in this way. In the absence of amplification products, certain parameters were altered (described in appendix 6.3).

2.5.5 THE EFFECT OF RNA ON RAPD-PCR BANDING PATTERNS

RAPD-PCR using random primer 970-11 (table 2.2) was performed as explained above (section 2.5) using DNA (extracted as described in section 2.4.1), from *P. intermedia* MH3 and RNA free DNA (as described in section 2.4.1.1).

2.5.6 STABILITY OF EXTRACTED DNA AND REPRODUCIBILITY OF AMPLIFICATION OF DNA

DNA was extracted (as described in section 2.4.1) from *P. intermedia* strain MH3 at monthly intervals for 7 months and stored at 4°C. Freshly extracted and previously extracted stored DNA was subjected to RAPD-PCR using random primer L10 as explained in table 2.3. Banding patterns were compared visually to assess the stability of extracted DNA over a 15 month period (table 2.3).

2.5.7 EFFECT OF DNA TEMPLATE CONCENTRATION OF RAPD-PCR AMPLIFICATION PROFILES

DNA (1 μl) extracted from *P. nigrescens* LM94 was quantified as described in section 2.4.3 and diluted with ultrapure water 1:2, 1:5, 1:10, 1:15, 1:20, 1:25, 1:50, 1:75, 1:100 and

1:200 to give a range of DNA concentrations. These dilutions plus the undiluted template (5 µl) were used for RAPD-PCR with primer L10 as described in section 2.5 to assess the effect of template concentration on the amplification profile.

2.5.8 RAPD-PCR OF *P. INTERMEDIA* AND *P. NIGRESCENS* DNA

RAPD-PCR was performed as described above (section 2.4). Four primers were tested; 970-11; Reverse sequencing primer, RSP; Universal sequencing primer, US and L10. Primers RSP, 970-11 and US were tested on a limited number of *P. intermedia* and *P. nigrescens* strains (*P. intermedia* ATCC 25261; MH15; MH6; MH12 and *P. nigrescens* LM94; MH4) and primer L10 was used to amplify DNA from all strains of *P. intermedia* and *P. nigrescens* listed in table 2.1. All primers were synthesised by the Oligonucleotide Synthesising Service, School of Biological Sciences, University of Manchester and L10 was also synthesised by Perkin Elmer.

2.5.9 RAPD-PCR OF DNA FROM OTHER MICROORGANISMS

DNA from PINLO strains, *P. corporis*, *P. pallens* and Gram-negative black-pigmenting clinical isolates classified as *P. intermedia* by Rapid ID 32 A, was subjected to RAPD-PCR as described (section 2.5) using only primer L10 (table 2.2). Amplification products were separated in 1.8% agarose gels as described in section 2.5.3 with amplification products from known strains of *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 25261 and ATCC 33563 (section 2.5.7).

Table 2.2 Sequence information and published sources of arbitrary primers used for RAPD-PCR analysis of *P. intermedia* and *P. nigrescens*.

PRIMER	NUCLEOTIDE SEQUENCE	LENGTH	PUBLISHED SOURCE
970-11	GTAAGGCCG	9 bases	Ménard *et al.*, 1992
L10	GTAGAGCTCGCGGCACTTG	19 bases	Corney *et al.*, 1993
RSP	GGAAACAGCTATGACCATGA	20 bases	Welsh *et al.*, 1992
US	GTAAAACGACGGCCAGT	17 bases	Corney *et al.*, 1993

Table 2.3 DNA extractions and RAPD-PCR reactions performed over a 15 month period to asses the stability of extracted DNA by reproducibility of RAPD-PCR banding profiles

MONTH	DNA EXTRACTION	RAPD-PCR OF DNA EXTRACTIONS
I	Yes	Month I
II	Yes	Months I,II
III	Yes	Months I, II, III
IV	Yes	Months I, II, II, IV
V	Yes	Months I, II, III, IV, V
VI	Yes	Months I, II, III, IV, V, VI
VII	Yes	Months I, II, III, IV, V, VI, VII
XV	No	Months I, II, III, IV, V, VI, VII

2.5.10 STATISTICAL ANALYSIS OF RAPD-PCR RESULTS

Hierarchical cluster analysis was used to group the microorganisms into species according to the RAPD-PCR banding pattern produced with primer L10. Three sets of comparisons were made (i) known strains of *P. intermedia* and *P. nigrescens*. (ii) black-pigmenting clinical isolates identified as *P. intermedia* in comparison with known strains of *P. intermedia* and *P. nigrescens*. (iii) PINLOs in comparison with known strains of *P. intermedia* and *P. nigrescens*, black-pigmenting clinical isolates, *P. pallens* and *P. corporis*. Advice and guidance concerning cluster analysis was provided by Dr. V. Hillier, Department of Medical Computation, University of Manchester.

2.5.10.1 *P. intermedia* and *P. nigrescens* DNA

After electrophoresis, the approximate sizes (bp) of the reproducible bands to be scored was determined (see appendix 6.4, table 6.1 A). The reproducible bands were scored visually as 1 (present) or 0 (absent) for each strain of *P. intermedia* and *P. nigrescens* and a binary matrix constructed. These data allowed hierarchical cluster analysis to be performed (SPSS for Windows V. 6.1) and a dendrogram to be constructed, using average linkage between groups (also called UPGMA or unweighted pair-group method using arithmetic averages) and the simple matching coefficient.

2.5.10.2 Gram-negative black-pigmenting clinical isolates

After electrophoresis, the approximate sizes (bp) of the reproducible bands to be scored was determined (see appendix 6.4, table 6.1 B).

The presence or absence of bands was scored in relation to those bands scored previously for *P. intermedia* and *P. nigrescens* and a new binary matrix constructed. Cluster analysis was performed as previously described (section 2.5.9.1).

2.5.10.3 PINLO strains, *P. corporis* and *P. pallens*

The presence or absence of bands was scored in relation to those bands scored previously for *P. intermedia*, *P. nigrescens* and the black-pigmenting clinical isolates labelled as *P. intermedia* (see appendix 6.4, table 6.1 C). A new binary matrix constructed with band information for all species. Cluster analysis was performed as previously described (section 2.5.9.1).

2.6 PARTIAL 16S rRNA SEQUENCE ANALYSIS

The following method describes how automated partial 16S rRNA gene sequencing was performed to achieve a 16S rDNA sequence. The procedure was identical for all microorganisms; *P. intermedia, P. nigrescens,* PINLO strains, Gram-negative black-pigmenting clinical isolates, other Gram-negative clinical isolates and Gram-positive clinical isolates.

2.6.1 PCR OF 500 BASE PAIR VARIABLE FRAGMENT FROM 16S rRNA GENE

DNA (2 µl extracted as described in section 2.4) was subjected to PCR in a Crocodile II™ thermal cycler (Appligene). The reaction mix had a total volume of 50 µl which consisted of *Taq* polymerase (1.5 units), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), $MgCl_2$ (1.5 mM), 1 x buffer IV and 0.3 µM of both primers; RE-RTU3 and RE-TPU1 (Choi *et al.*, 1994; table 2.4). Both primers were synthesised by the Oligonucleotide Synthesising Service, School of Biological Sciences, University of Manchester. The reaction mix was covered by an equal volume (50 µl) of mineral oil to prevent evaporation before subjecting to PCR. Negative controls consisted of all components of reaction mix other than bacterial template DNA.

The thermal cycling profile was as follows;

1 cycle of 4 min at 94°C, 1 min at 55°C, 1 min at 72°C

29 cycles of 1 min at 94°C, 1 min at 57°C, 1 min at 72°C

1 cycle of 1 min at 94°C, 1 min at 57°C, 5 min at 72°C.

2.6.2 ELECTROPHORESIS OF PCR PRODUCT

PCR products (3 µl) were resolved in 1.0% agarose gels at 60V for 1-2 h, stained with ethidium bromide and visualised under ultraviolet light (λ = 254 nm). A λ *Pst* I digest was used as a size marker (270 ng/µl). Gels were photographed on Polaroid 665 and 667 films and the presence of a approximate 500 bp fragment confirmed.

2.6.3 PURIFICATION OF PCR PRODUCT

QIAquick PCR Purification Kit (Qiagen) was used to clean the PCR product according to the manufacturer's instructions. Details of the QIAquick PCR Purification Kit can be found in appendix 6.2.2.

2.6.4 QUANTIFICATION OF AMOUNT OF DNA

The concentrations of DNA in each 500 bp fragment were estimated by visual comparison with a known amount of λ DNA (4.48 ng). Purified PCR products (5 µl) were resolved in 0.8% agarose gels at 60 V for 1 h, with a λ *Pst* I digest (270 ng/µl). Gels were stained in ethidium bromide (1 µg/ml in 1 x TPE), visualised under ultraviolet light (λ = 254 nm) and photographed on Polaroid 665 and 667 films. The DNA preparations were diluted in water where appropriate to obtain approximately concentrations in the range 30-90 ng for each sample.

2.6.5 CYCLE SEQUENCING REACTIONS

Purified PCR products were subjected to PCR in a Perkin Elmer model 2400 thermal cycler. The reaction mix had a total volume of 20 µl which consisted of 8 µl ABI Prism™ terminator cycle sequence ready reaction mix (*P. intermedia*, *P. nigrescens* and PINLO strains) or 4 µl ABI Prism™ BigDye™ terminator cycle sequence ready reaction mix (Gram-negative black-pigmenting clinical isolates, other Gram-negative clinical isolates and Gram-positive clinical isolates) and either RE-RTU3 or RE-TPU1 (0.15 µM). Two reaction mixes were set up for each PCR product, one with each primer. PCR product, the amount adjusted according to concentration, was added and the volume made up with water. The thermal cycling profile was as follows; 25 cycles of 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C.

2.6.6 ETHANOL PRECIPITATION OF PRODUCTS

After cycle sequencing, all 20 µl of product was added to sodium acetate (2 µl; final concentration 0.1 M; pH 5.2) and 100% v/v ethanol (50 µl). Incubation at 0°C for 10 min was followed by centrifugation (13,000 rpm for 25 min.) The pellet was washed in 70% v/v ethanol and allowed to dry thoroughly.

2.6.7 AUTOMATED SEQUENCING OF 16S rDNA

2.6.7.1 *P. intermedia* and *P. nigrescens* strains

This was performed by Lawrence Hall, University of Manchester and results received in computer file for subsequent analysis.

2.6.7.2 PINLO strains, Gram-negative black-pigmenting clinical isolates, other Gram-negative clinical isolates and Gram-positive clinical isolates

Dry cycle sequencing products were sent to Oswals DNA service, University of Southampton. Results were received in computer file for subsequent analysis.

2.6.8 SEQUENCE DATA ANALYSIS

ABI Prism™ DNA Sequencing Analysis Software (Perkin Elmer) v.2.1.2 was used to view, check and edit sequences. Sequences were manipulated using the Genetics Computer Group (GCG) package, unix version 8.0, (1994), Wisconsin, USA. Sequences in text file format were entered and edited using the SEQED command and the REFORMAT command was used to put the sequence into 'gcg' format. Sequences resulting from amplification with primer RE-RTU3 were reversed and complemented using the REVERSE command. Results from each primer were then aligned using the GAP function and double checked against each other to produce a consensus sequence. A FASTA search was performed using the partial 16S rRNA sequence to find the most likely phylogenetic relationship. This is summarised in appendices 6.8.1-6.8.3 and illustrated in appendices 6.8.3.1-6.8.3.5.

2.6.8.1 Sequence data analysis of *P. intermedia* and *P. nigrescens* and Gram-negative black-pigmenting clinical isolates

Sequences were entered and edited as described in section 2.6.8.

As a result of the FASTA search, consensus sequences were compared to information available for the type strains *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563 and *P. nigrescens* ATCC 25261 within the EMBL database (appendix 6.6). The DISTANCES program was used to asses the distances between the informative positions in the rDNA sequences of the test organisms and *P. intermedia* and *P. nigrescens* in the EMBL database. LINEUP was used to remove regions of insertions or deletions and trim the edges of the aligned sequences prior to calculating distances.

2.6.8.2 Sequence data analysis of PINLO strains

Sequences were entered and edited as described in section 2.6.8.

A FASTA search was performed and as a result, the consensus sequence was compared to information available for the type strains *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563 and *P. nigrescens* ATCC 25261, *P. corporis*, *P. denticola* and *P. pallens* within the

EMBL database (appendix 6.6). The DISTANCES program was used to asses the distances between the informative positions in the rDNA sequences of the PINLO strains and the other *Prevotella* species.

2.6.8.3 Sequence data analysis of Gram-negative non-black-pigmenting clinical isolates and Gram-positive clinical isolates

Sequences were entered and edited as described in section 2.6.8 and a FASTA search was performed to find the most likely phylogenetic relationship.

2.7 DESIGN OF *P. INTERMEDIA* AND *P. NIGRESCENS* SPECIFIC OLIGONUCLEOTIDES

The EMBL DNA database at Seqnet Daresbury was accessed and used to provide 16S rRNA gene sequence information for *P. intermedia* and *P. nigrescens*. This information was carefully analysed to find species-specific regions that could be exploited as unique PCR primers specific for each species.

2.7.1 USE OF EMBL DNA DATABASE TO ACCESS 16S SEQUENCE INFORMATION FOR *PREVOTELLA* SPECIES

The EMBL DNA Database was accessed via Seqnet Daresbury. A search was performed using keywords for 'Sequence-all text': *Prevotella* and 'Sequence-features': 16S. Forty-seven entries matched these search criteria. Five complete 16S rRNA sequences were found for *P. intermedia* ATCC 25611 (L16468 and X73965), *P. nigrescens* NCTC 9336 (X73963), *P. nigrescens* ATCC 33563 (L16471) and *P. nigrescens* ATCC 25261 (L16479). The accession numbers are shown in parentheses.

These sequences were aligned and compared using the command PILEUP. The command PRETTY with a plurality of 5, produced a consensus sequence for all five chosen entries.

2.7.2 SELECTION OF UNIQUE SEQUENCES FOR *P. INTERMEDIA* AND *P. NIGRESCENS*

Regions were there was no consensus sequence between *P. intermedia* and *P. nigrescens* were analysed for suitable regions to be exploited for PCR primer construction. 16 potential regions of lengths in the range of 19-36 bases were selected. Each was then analysed for suitability as described below.

2.7.2.1 Comparison of potential primer sequences to 16S rRNA gene sequences of other oral bacterial species

Sequences from other oral bacteria found within the EMBL database were aligned and compared by Seqnet, Daresbury. The organisms are listed in table 2.4 and included *P. melaninogenica*, *P. gingivalis*, *B. forsythus*, *F. nucleatum*, *A. actinomycetemcomitans*, *T. denticola* and *T. socranskii* and the location of the candidate oligonucleotides checked for lack of homology to any of them.

2.7.2.2 Formation of stemloop structures

The formation of undesirable inverted repeats; stemloops, by candidate sequences was checked using the STEMLOOP program. The possible occurrence of stemloops was considered undesirable.

2.7.2.3 FASTA search

The candidate sequences were compared to all entries in the EMBL database by performing a FASTA search. The output list of sequences was studied and those displaying a high degree of homology to other *Prevotella* species, other putative periodontopathogens, resident oral microorganisms or relevant human sequences were discarded. Potential regions with little homology to other bacterial species were chosen. Regions of homology to other sequences were tolerated if mismatches within this identity occurred in the centre or throughout the length of the sequence rather than at the ends (Dieffenbach *et al.*, 1993).

2.7.3 SPECIES-SPECIFIC OLIGONUCLEOTIDES

Two unique sequences were decided upon; designated P-int and P-nig. They were synthesised by the oligonucleotide synthesising service (University of Manchester, Manchester, UK). The sequences are given in table 2.4.

Table 2.4 Sequence information and published sources (where relevant) of PCR primers for the specific identification of *P. intermedia* or *P. nigrescens*

SEQUENCE NAME	SEQUENCE	LENGTH (BASES)	SPECIES
P-int	CACGTCAGATGCCATATGTGGACA	24	*P. intermedia*
P-nig	TCCCTTACCGGAAAACCTTTG	21	*P. nigrescens*
1Bi-1	GCATTTACCCTTCGAATAAGGACC	24	*P. intermedia*
2Bi-1	TGTGCGCCATTGCATGTACCTCAT	24	*P. nigrescens*
RE-TPU1	AGAGTTTGATCMTGGCTCAG	20	universal
RE-RTU3	GWATTACCGCGGCKGCG	18	universal

2.7.4 PUBLISHED *P. INTERMEDIA* AND *P. NIGRESCENS* SPECIES-SPECIFIC OLIGONUCLEOTIDES

Two regions of 16S rRNA that had been previously exploited as species-specific oligonucleotide probes and PCR primers were analysed. (Shah *et al.*, 1995; Mättö *et al.*, 1996). These were 1Bi-1 (homologous to *P. intermedia*) and 2Bi-1 (homologous to *P. nigrescens*) (Dix *et al.*, 1990). The published sequence was located within the consensus sequence produced by the PRETTY command (as described in section 2.7.1). These sequences are detailed in table 2.4. They were synthesised by the oligonucleotide synthesising service (University of Manchester, Manchester, UK) and examined in parallel with the unique sequences designed (section 2.8).

2.8 USE OF SPECIES-SPECIFIC OLIGONUCLEOTIDES AS PCR PRIMERS

Each species-specific oligonucleotide was used as one of a primer pair with RE-TPU1 for PCR amplification of *P. intermedia* or *P. nigrescens* DNA.

2.8.1 SPECIES-SPECIFIC PCR

PCR was carried out in a Crocodile II™ thermal cycler (Appligene) under the following conditions. The reaction mix had a total volume of 50 µl which consisted of Qiagen *Taq* polymerase (1.5 units), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1 x Qiagen buffer and 0.3 µM of both primers; RE-TPU1 (Choi *et al.*, 1994) and test primer. Each primer 1Bi-1, 2Bi-1, P-int and P-nig was used with RE-TPU1 as the forward primer. The reaction mix was overlaid by an equal volume (50 µl) of mineral oil to prevent evaporation before subjecting to PCR. Negative controls consisted of all components of reaction mix other than bacterial template DNA.

All reactions were performed with 2 µl template DNA from *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 25261, *P. nigrescens* ATCC 33563, *P. gingivalis* ATCC 33227, *A. actinomycetemcomitans* ATCC 29525 and *C. ochracea* W42 unless otherwise stated.

2.8.2 NEGATIVE CONTROLS

DNA was extracted (as described in section 2.4.1 for Gram-negative bacteria) from *P. gingivalis* ATCC 33227, *C. ochracea* W42 and *A. actinomycetemcomitans* ATCC 29525 for use as internal negative controls to test the specificity of the oligonucleotide primers during all optimisation experiments. In addition negative controls consisting of all

components of reaction mix other than template DNA were included, with sterile water included instead of template.

2.8.3 OPTIMISATION OF PCR REACTION

Optimisation reactions were undertaken for all 4 primers. The template DNA used was 40-100 ng of *P. intermedia* ATCC 25261, *P. nigrescens* ATCC 33563 and *P. nigrescens* ATCC 25611 in addition to the negative control organisms described in section 2.8.2 above. Once a satisfactory result had been obtained, the PCR reaction was repeated so that each PCR reaction was performed on at least 2 separate occasions.

2.8.3.1 Annealing temperature

The PCR reaction was carried out using a range of annealing temperatures. A temperature of 57°C was used initially (section 2.6.1). PCR was subsequently tested raising the annealing temperature in 3°C increments (between 57°C and 69°C) and finally by 1°C to achieve specificity. The final thermal cycling profiles were as follows;

1 cycle of 4 min at 94°C, 1 min at R°C, 1 min at 72°C

29 cycles of 1 min at 94°C, 1 min at R°C, 1 min at 72°C

1 cycle of 1 min at 94°C, 1 min at R°C, 5 min at 72°C.

Where R = 67°C for primer P-int, 65°C for primer P-nig, 68°C for primer 1Bi-1 and 69°C for primer 2Bi-1.

2.8.3.2 Magnesium and primer concentrations

The optimum magnesium and primer concentrations were determined using a titration series. PCR was carried out as previously described (section 2.8.1) using $MgCl_2$ concentrations in the range 1.5 mM - 5 mM and primer concentrations in the range 0.1 μM - 0.5 μM.

2.8.4 ELECTROPHORESIS OF PCR PRODUCTS

PCR products (5 μl) were separated in 1.0 % agarose gels at 60V for 1-2h, stained with ethidium bromide and visualised under ultraviolet light (λ = 254 nm). A ϕx174 DNA - *Hae* III digest was used a size marker. Gels were photographed on Polaroid 665 and 667 film and examined for the occurrence of the correct bands (approximately 204 bp P-int; 228 bp P-nig; 499 bp 1Bi-1; 488 bp 2Bi-1).

2.8.5 AMOUNT OF DNA TEMPLATE

All four primers were tested as described in section 2.8.1, using optimal conditions as determined in section 2.8.3 against approximately 20 ng, 40 ng, 60 ng, 80 ng and 100 ng template DNA. See section 2.4.3 for quantification of DNA. The template DNA used was as described in section 2.8.1. PCR products were separated by electrophoresis as described in section 2.8.4. All detection limit PCRs were repeated three times.

2.8.6 SPECIFICITY OF PCR REACTION

DNA from known strains of *P. intermedia* and *P. nigrescens*, 4 Gram-negative black-pigmenting clinical isolates, *P. gingivalis* ATCC 33227, *C. ochracea* W42, *A. actinomycetemcomitans* ATCC 29525, *P. pallens*, *P. corporis* and PINLO strains A391, HST 2160 and HST 1156 was extracted (as described in section 2.4.1 for Gram-negative bacteria) and used on 2 separate occasions to test the specificity of the oligonucleotide primers. PCR was performed as described (section 2.8.1) using conditions determined as described in section 2.8.3. Primers P-int, P-nig and 1Bi-1 were tested using approximately 40 ng of template DNA from these strains. PCR products were separated by electrophoresis as described in section 2.8.4.

2.8.7 MULTIPLEX PCR

2.8.7.1 Multiplex PCR set-up

PCR was performed with primers P-nig and 2Bi-1 under the optimal conditions described above (2.8.3) with the addition of another set of primers. Each PCR reaction included the test primer (P-nig, or 2Bi-1) plus RE-TPU1 as one primer pair and the universal sequencing primers RE-TPU1 and RE-RTU3 (section 2.6.1; Choi *et al.*, 1994) as the other. All primers were used at 0.3 μM. DNA from *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *P. nigrescens* ATCC 25261 and *P. gingivalis* was used as template. Two PCR reactions were performed for each organism, one using approximately 40 ng of template DNA and one using approximately 80 ng of template DNA. Multiplex PCR reactions were performed using equal amounts of all four primers.

2.8.7.2 Electrophoresis of PCR products

PCR products were separated as described in section 2.8.4. The number and sizes of the PCR products was noted.

2.8.8 VARIATIONS IN AMPLIFICATION AS A RESULT OF INCONSISTENCIES IN TEMPERATURE PROFILE ACROSS PCR THERMAL CYCLER

The consistency of amplification was tested by placing 8 PCR reactions in specified sites within the Crocodile II™ thermal cycler (Appligene). Amplification with primers P-int (template 40 ng of *P. intermedia* ATCC 25611) and P-nig (template 40 ng of *P. nigrescens* ATCC 25261) was tested independently this way. PCR was set up as described in section 2.9.1.

MATERIALS AND METHODS

SECTION B: CLINICAL MICROBIOLOGY (PAPILLON-LEFÈVRE SYNDROME; PLS)

2.9 IDENTIFICATION OF BACTERIAL CLINICAL ISOLATES FROM THE PERIODONTAL POCKET OF PATIENTS WITH PLS

2.9.1 PATIENTS

Two patients with Papillon-Lefèvre Syndrome attended the University of Manchester, Dental Hospital, Higher Cambridge Street, Manchester in June 1997. (See appendix 6.13 for patient information).

2.9.2 SAMPLING OF THE PERIODONTAL POCKET

Samples were taken from the gingival pocket by Dr. J. James (Lecturer in Periodontology, Turner Dental School, University of Manchester). Patient 1 had only six remaining teeth and samples were taken from the gingival pocket of each one. Samples from patient 2 were taken from the gingival pockets of molars and canine teeth. Scrapings were placed into reduced transport fluid (prepared by Dr. M. Abdi, University of Manchester) for rapid transportation to Manchester Royal Infirmary (MRI) where pure cultures of isolates were obtained and Gram stain morphology determined. Periodontal pocket material was also smeared onto glass microscope slides and retained for subsequent analysis (see section 2.11.6). Additional samples were resuspended in PBS containing 0.01% thiomersal, frozen and sent to the University of Queensland, Brisbane, Australia for monoclonal antibody studies. Full details (methodology and results) of the work carried out by Dr. S. Hamlet at the University of Queensland can be found in appendix 6.24.

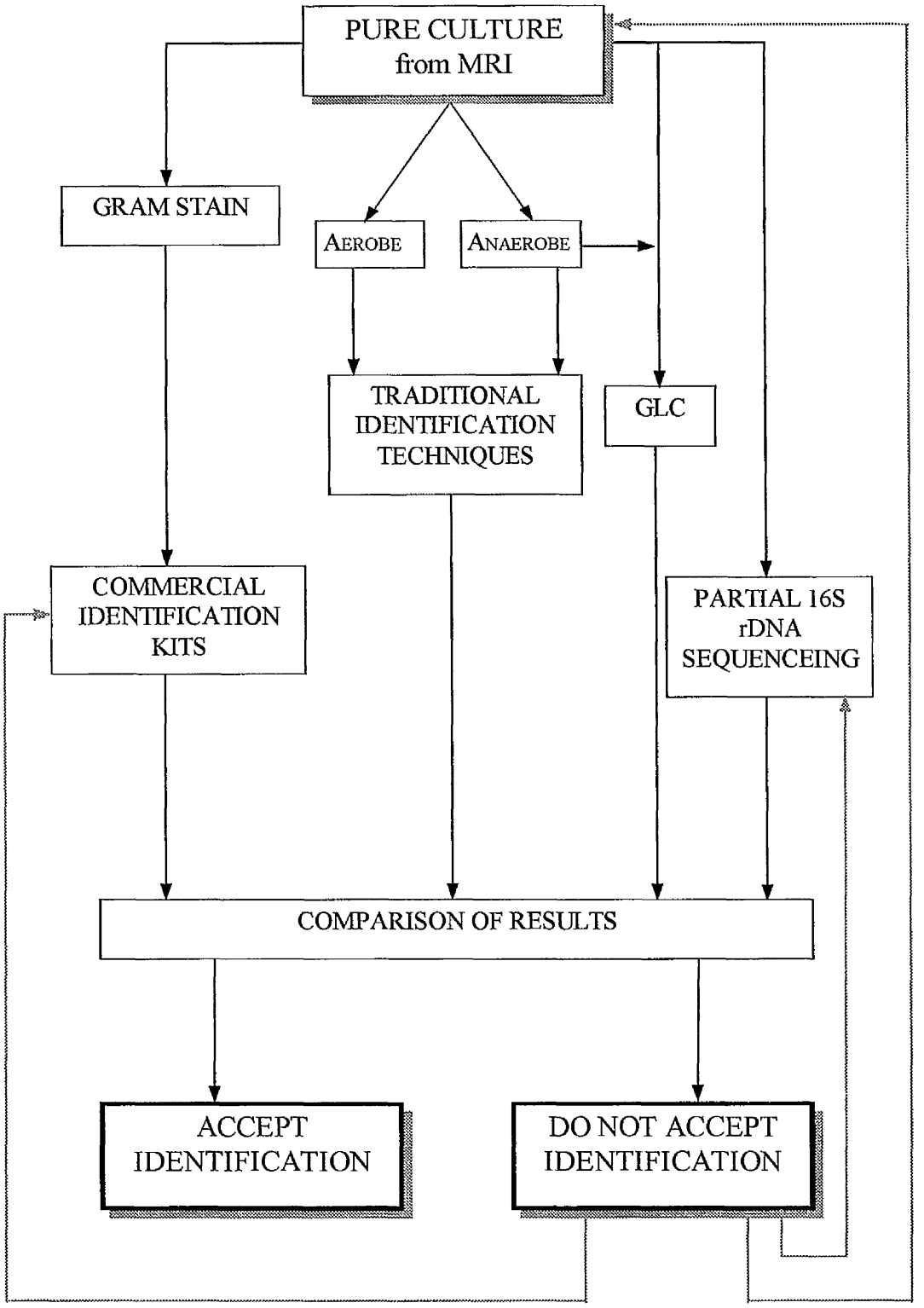
2.9.2.1 Labelling of samples

Samples were labelled 1 or 2 according to which patient the sample was taken from and labelled A-F in order of sampling, for example, sample *2B* came from patient 2 site B, the second site to be sampled. A second number was added in front by staff at MRI whilst obtaining pure culture of the isolate, for example *2B* became **9** *2B*.

2.9.3 INITIAL CHARACTERISATION OF ISOLATES

Organisms were cultivated from samples, purified and Gram stained by Malcolm Armstrong at Manchester Royal Infirmary (MRI). The pure colonies were returned to Manchester University for identification as described in sections 2.10 and 2.11. The steps taken in the identification of clinical isolates are summarised in figure 2.1.

Figure 2.1 Flow diagram summarising methods used to identify clinical isolates



2.10 IDENTIFICATION OF AEROBIC (AND FACULTATIVE) MICROORGANISMS FROM PATIENTS WITH PLS

2.10.1 COMMERCIAL IDENTIFICATION KITS

A range of Commercial identification kits (API 20 NE; API NH, Rapid ID 32 Strep; API Coryne; Rapid ID 32A; Rapid ANA II) were used to assist identification. The identification kit used was determined by the Gram stain result and morphology as performed by staff at Manchester Royal Infirmary. API 20 NE was used to identify Gram-negative rods which do not belong to the Enterobacteriaceae such as *Pseudomonas*, *Acinetobacter* and *Vibrio* species, API Coryne for the identification of coryneform bacteria, API NH for the identification of *Neisseria*, *Branhamella* and *Haemophilus*, Rapid ID 32 Strep for the identification of streptococci and Rapid ID 32 A and RapID ANA II for the identification of facultative species. All identification kits were used according to the manufacturer's instructions. Reagents and reactions are tabulated in appendices 6.14 and 6.15.

Microcodes generated using API 20 NE, API NH, Rapid ID 32 Strep, API Coryne or rapid ID 32A were sent for analysis to bioMérieux. Microcodes generated using RapID ANA II were sent to Pro-Lab Diagnostics, for identification.

2.10.2 SUBSEQUENT IDENTIFICATION

Isolates producing inconclusive results using one or more identification kits were subjected to further tests. All isolates were tested for catalase and oxidase activity, oxidation-fermentation characteristics and growth in an anaerobic environment (see sections 2.10.2.1 to 2.10.2.10 below). A positive catalase reaction was the formation of immediate bubbles, whilst absent or isolated bubbles was considered a negative test result (Summanen *et al.*, 1993). Additional tests were used in some cases (sections 2.10.2.6 - 2.10.2.10). Media and reagents used in sections 2.10.2.2, 2.10.2.3, 2.10.2.4 and 2.10.2.8 are detailed in appendix 6.16.

2.10.2.1 Gram stain and morphology in BHI broth

Gram stain results were supplied by MRI, however in the majority of cases they were checked. Clinical isolates were Gram stained according to the method devised by Christian Gram in 1884. Colonies grown in BHI liquid medium were smeared over the slide, the smears are air dried and heat fixed. The slide was flooded with crystal violet for 30 s, which was washed off with running tap water. This was then followed with Lugol's iodine applied for 30 s. The smear was washed with acetone and rinsed with water before flooding with

the counterstain safranin for thirty seconds. The slide was rinsed and dried. Light microscopy used a x100 oil immersion objective lens to visualise the slides. The isolate was classified as Gram-negative (stained red) or Gram-positive (stained purple) accordingly. The shape and relative size of cells was noted.

2.10.2.2 Catalase test

All clinical isolates were tested for catalase production. A few drops of 3% hydrogen peroxide was added to the surface of a single colony grown on a FAA plate. The petri dish lid was replaced and the colony observed for the production of effervescence, indicating a positive reaction. The production of catalase was tested for on two separate occasions.

2.10.2.3 Test for oxidase activity

This was performed as described in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993). Three drops of oxidase reagent (see appendix 6.16.2) were pipetted onto filter paper in a petri dish. A sterile plastic loop was used to smear the test culture across the paper. A positive reaction was indicated by the development of a purple colour within 30 s. Oxidase activity was tested for on two separate occasions.

2.10.2.4 Oxidation or fermentation of glucose (Hugh and Leifson)

Duplicate universals of Hugh and Leifson's oxidation-fermentation medium (see appendix 6.16.3) were inoculated for each test organism by stabbing with a straight wire. One universal was overlaid with a layer of 1cm of paraffin. They were incubated at 37°C for two weeks.

2.10.2.5 Growth in an anaerobic environment

The ability of isolates to grow in anaerobic environment of hydrogen, carbon dioxide and nitrogen (10:10:80% v/v) was tested. The test isolated was used to inoculate a CBA plate which was incubated under anaerobic conditions in an anaerobic cabinet for 3-5 days.

2.10.2.6 Colony colour

In the case of those clinical isolates exhibiting highly coloured colonies, the colour was noted where it aided identification.

2.10.2.7 Extracellular polysaccharide production

The detection of (dextran, levan) production was used to aid differentiation of some of those clinical isolates identified as streptococcal species using Rapid ID 32 Strep. Organisms were grown on TYC agar (prepared as described in appendix 6.1.3.3) for 5-10 days at 37°C and examined frequently under a plate microscope for the appearance of extracellular polysaccharide.

2.10.2.8 Nitrate reduction

This was performed as described in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993). 1 ml of 0.1% (w/v) KNO_3 in nutrient broth was warmed to 37°C and inoculated with a loopful of test organism. Following 15 min incubation at 37°C, 3 drops of each of solutions A and B were added (for details of reagents see appendix 6.16.4). The development of a pink colour indicated nitrate reduction.

2.10.2.9 Resistance to optochin antibacterial

The sensitivities of those isolates given the presumptive identification of *Streptococcus pneumoniae* to the antibacterial optochin was tested. Isolates were inoculated onto a CBA plate (appendix 6.1.3.2) and a paper disc impregnated with optochin (obtained from Microbiology Department, Manchester Royal Infirmary) was placed in the centre of the heavy inoculum. Agar plates were inverted and incubated at 37°C for 7 days after which time they were examined for a zone of inhibition.

2.10.2.10 V-factor

A requirement for V-factor (co-dehydrogenase NADH) was tested by observing for enhanced growth and satellitism of bacteria in the presence of *Staphylococcus aureus*.

2.10.3 PARTIAL 16S RRNA GENE SEQUENCING

Clinical isolates which were not identified satisfactorily were analysed by partial 16S rRNA gene sequencing as described in section 2.6 following DNA extraction as described in section 2.4.

2.10.3.1 Analysis of sequencing information

The partial 16S rDNA sequences obtained were analysed as described in section 2.6.8 and illustrated in appendix 6.8.

2.10.4 FINAL IDENTIFICATION OF AEROBIC (AND FACULTATIVE) CLINICAL ISOLATES

All results obtained by methods described in sections 2.10.1-2.10.3 above were cross referenced and checked. Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) was used to assist identification.

2.11 IDENTIFICATION OF ANAEROBIC (AND FACULTATIVE) MICROORGANISMS FROM PATIENTS WITH PLS

2.11.1 COMMERCIAL IDENTIFICATION KITS

Two commercial identification kits; Rapid ID 32 A and RapID ANA II were used to assist identification. Reactions and reagents are tabulated in appendices 6.14 and 6.15. The Gram stain result and morphology as performed by staff at MRI determined the identification kit used.

2.11.2 SUBSEQUENT IDENTIFICATION

Isolates producing inconclusive results using one or more identification kits were subjected to further tests; Gram stain and Morphology in BHI broth although supplied by MRI was checked in the majority of cases (as described in section 2.10.2.1). Catalase (as described in section 2.10.2.2), oxidase activity (as described in section 2.10.2.3) and growth in an aerobic environment (section 2.11.2.1 below) were tested in all cases. Colony colour (as described in section 2.10.2.6) was noted in some cases.

In addition, GLC (section 2.11.3) and partial 16S rRNA gene sequencing (section 2.11.4) were performed in some cases.

2.11.2.1 Growth in an aerobic environment

In addition to identification tests already described, anaerobic and facultative organisms were tested for the ability to grow in an aerobic environment, confirming their facultative nature. Isolates were grown on FAA plates (appendix 6.1.3.1) at 37°C for 5 days.

2.11.3 GAS-LIQUID CHROMATOGRAPHY (GLC)

Obligate and facultative anaerobic isolates were examined using Gas-Liquid Chromatography (GLC). Each culture supernatant was tested for volatile and non-volatile products twice. Sterile GLC broth was also treated and examine in the same way.

2.11.3.1 Growth of clinical isolates for GLC analysis

Bacterial species were grown in GLC broth (appendix 6.1.4.3) for 24-48 h after which time the culture was Gram stained and the culture supernatant harvested for analysis.

2.11.3.2 Analysis of volatile fatty acids and alcohols

2.11.3.2.1 Preparation of volatile acid standard solutions

Volatile acid standards were prepared as described in the 4th edition of the *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977). Stock solutions (100 ml aqueous) were prepared (table 2.5). A working mixed standard was made by adding a volume of 1ml from each stock solution into a total volume of 100 ml. An ether extract of this was prepared and analysed as described in section 2.11.3.2.3.

2.11.3.2.2 Preparation of alcohol standard solutions

Alcohol standards were prepared as described in the *Anaerobe Laboratory Manual* 4th Edition (Holdeman *et al.*, 1977). A working mixed standard was prepared as described above in section 2.11.3.2.1 using stock solutions (100 ml aqueous) prepared as shown in table 2.6. An ether extract of the mixed working standards was prepared and used as described below (section 2.11.3.2.3).

2.11.3.2.3 Preparation of ether extract

Diethyl ether (1 ml), 50% (v/v) H_2SO_4 (0.2 ml) and sodium chloride (0.4 g) was added to 1 ml culture supernatant or working standard and mixed thoroughly. After centrifugation (2 min at 3,000 rpm), the ether layer was removed and anhydrous 4-20 mesh calcium carbonate (0.25 g) was added to this layer.

2.11.3.2.4 Injection of sample

A volume (2 µl) of ether extract was injected onto the column. The syringe was flushed through with ether between injections in order to remove traces of the previous sample.

2.11.3.2.5 Retention times of volatile acids and alcohols

The temperature program was used as described in section 2.11.3.2.6. An ether extract of the working standard was prepared as described in section 2.11.3.2.3 and injected onto the column. The retention times were noted and the order of elution matched to the typical chromatograms in the *Anaerobe Laboratory Manual* 4th Edition (Holdeman *et al.*, 1977).

2.11.3.2.6 Final analysis conditions

The injector temperature was 150°C, the detector was 250°C, the initial temperature of the column was 65°C for 30 s increasing to 115°C over 2 min and maintaining this temperature for a further 6 min and 20 s. The equilibration time between injections was 4 min. The FID was set at 10 (low sensitivity range for trace elements) with an attenuation of 128.

The gas flow rates were as follows:

air 300 ml / min; hydrogen 30 ml / min; nitrogen 30 ml / min

Bacterial culture supernatants from anaerobic and facultative isolates were analysed for the presence of volatile acids and alcohols using this profile.

Peaks were recorded visually using a Kipp and Zonen BD8 Multirange chart recorder and the retention times recorded manually. The times of peaks produced by compounds of bacterial fermentation were compared to the times of the standard solutions to indicate what each peak represented.

2.11.3.2.7 Column packing

The glass column was already packed with 6% Carbowax 20 MTPA on Chromosorb W AWDMCS, purchased originally (Jenkins, 1988) from Phase Separations (Wales, UK).

2.11.3.3 Analysis of non-volatile acids

2.11.3.3.1 Preparation of non-volatile acid standard solutions

Non-volatile acid standards were prepared as described in the *Anaerobe Laboratory Manual* 4th Edition (Holdeman *et al.*, 1977). Stock solutions (100 ml aqueous) were prepared (table 2.7). A working mixed standard was made by adding a volume of 1 ml from each stock solution into a total volume of 100 ml.

2.11.3.3.2 Preparation of methyl derivatives of non-volatile acids

Methyl derivatives of non-volatile acids were prepared as follows. Methanol (2 ml) and 50% (v/v) H_2SO_4 (0.4 ml) was added to 1ml culture supernatant and incubated in a water bath at 60°C for 30 min. Water (1 ml) and chloroform (0.5 ml) was added and mixed thoroughly. After centrifugation (2 min at 3,000 rpm), 2 µl was drawn into the injection syringe directly from the lower chloroform layer.

2.11.3.3.3 Injection of sample

A volume of 2 µl of sample was drawn into the syringe from the chloroform layer and injected onto the column. The syringe was flushed through with chloroform between injections in order to remove traces of the previous sample.

2.11.3.3.4 Retention times of non-volatile acids

The same temperature program was used as described in section 2.11.3.2.6. The working standard was methylated as described in section 2.11.3.3.2 and injected onto the column. The retention times were noted and the order of elution matched to the typical chromatograms in the *Anaerobe Laboratory Manual* 4th Edition (Holdeman *et al.*, 1977).

2.11.3.3.5 Final analysis conditions

The temperature program used was as described in section 2.11.3.2.6.

All bacterial culture supernatants from anaerobic and facultative Gram-positive bacteria were analysed for the presence of non-volatile acids using this profile.

Table 2.5 Components of volatile acid stock solutions

VOLATILE ACID	AMOUNT OF VOLATILE ACID (ML)	AMOUNT OF DISTILLED WATER (ML)
Acetic	5.7	94.3
Propionic	7.5	92.5
Iso-butyric	4.6	95.4
n-Butyric	9.1	90.9
Iso-valeric	5.45	94.55
n-Valeric	10.9	89.1

Table 2.6 Components of alcohol stock solutions

ALCOHOL	AMOUNT OF ALCOHOL (ML)	AMOUNT OF WATER (ML)
Ethanol	10.0	90.0
Iso-propanol	3.5	96.5
n-Butanol	1.0	99.0

Table 2.7 Components of non-volatile acid stock solutions

NON-VOLATILE ACID	AMOUNT OF NON-VOLATILE ACID	AMOUNT OF DISTILLED WATER (ML)
Lactic	8.4 ml	91.6
Oxalic	6g	100.0
Malonic	5g	100.0
Succinic	6g	100.0

2.11.4 PARTIAL 16S RRNA GENE SEQUENCING

Clinical isolates not identified satisfactorily by the techniques mentioned above were subjected to partial 16S rRNA sequence analysis as described in section 2.6 following DNA extraction as described in section 2.4.

2.11.4.1 Analysis of sequencing information

The partial 16S rDNA sequences obtained were manipulated as described in section 2.6.8 and illustrated in appendix 6.8.

2.11.5 FINAL IDENTIFICATION OF ANAEROBIC (AND FACULTATIVE) CLINICAL ISOLATES

All results obtained by methods described in sections 2.11.1-2.11.4 above were cross-referenced and checked. Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) was used to assist identification.

2.11.6 DETERMINING THE PRESENCE OR ABSENCE OF SPIROCHAETES IN GINGIVAL SAMPLES

Gingival pocket material was stained using a modified Gram stain and viewed by light microscopy for the occurrence of spirochaetes. Smears of gingival plaque material on microscope slides (see section 2.9.2) were heat fixed and stained using crystal violet and Lugol's iodine as described in section 2.10.2.1. The counterstain used was 20% (w/v) carbol fuchsin. The slide was flooded with counterstain for 5 minutes, after which time it was rinsed off and the slide dried. Slides were viewed under a Zeiss (Germany), Axioplan light microscope at x1500 magnification with the help of Dr. H. Carter (Oral Pathology, Turner Dental School, University of Manchester).

2.12 FURTHER IDENTIFICATION OF ANAEROBES IDENTIFIED AS *P. INTERMEDIA* BY RAPID ID 32 A

Three clinical isolates from the Papillon-Lefèvre Syndrome patients were identified as *P. intermedia* using the Rapid ID 32 A kit. A gingival sample taken by Dr. J. James (Lecturer in Periodontology, Turner Dental School, University of Manchester) from a patient with gingival overgrowth was examined for *Prevotella* species and found to contain one isolate also identified as *P. intermedia* using the Rapid ID 32 A kit. Four methods previously described were used to determine whether these isolates were *P. intermedia* or *P. nigrescens* by comparison of results obtained with those obtained for known strains of *P.*

intermedia and *P. nigrescens*. DNA was extracted from these isolates as described in section 2.4.1, and amplified using random primer L10 (section 2.5.7). Extracted DNA was also used for partial 16S rRNA gene sequencing (section 2.6) and as a template for PCR (section 2.8.8). In addition the isolates were examined as detailed below.

2.12.1 COMMERCIAL IDENTIFICATION KITS

The isolates were originally identified by Rapid ID 32 A and were also examined using the RapID ANA II identification kit.

2.12.2 TRADITIONAL LABORATORY IDENTIFICATION TESTS

P. intermedia and *P. nigrescens* isolates were tested for catalase (section 2.10.2.2), oxidase (section 2.10.2.3) and grown in FAB before Gram staining (section 2.3.2.1). Strains were grown on egg yolk agar (section 2.3.2.1) to test for lipase activity.

2.12.3 GLC

The four isolates were grown in GLC broth (section 2.3.2.2) and analysed by GLC (for details see section 2.11.3).

2.13 FURTHER EXAMINATION OF PINLOS

As previously described, DNA extracted from three PINLOs; A391, HST 1156 and HST 2160 was used as a template for RAPD-PCR (see section 2.5.9), PCR (section 2.8.6) and for partial 16S rRNA gene sequence analysis (section 2.6). In addition the strains were examined as described for isolates of *P. intermedia* and *P. nigrescens* (section 2.12), using commercial identification kits RapID ANA II system and Rapid ID 32 A, traditional laboratory identification tests (catalase, section 2.10.2.2; oxidase, section 2.10.2.3; Gram stain, section 2.10.2.1 and lipase activity, 2.3.2.1) and grown in GLC broth (section 2.3.2.2) for GLC analysis (section 2.11.3).

3. RESULTS

3.1 GROWTH AND MAINTENANCE OF ANAEROBIC AND FACULTATIVE BACTERIAL SPECIES

3.1.1 SOLID MEDIUM

All black-pigmenting bacteria (*Prevotella intermedia*, *P. nigrescens*, *P. corporis*, PINLO and *Porphyromonas gingivalis*) grew successfully on FAA and CBA in an anaerobic environment (10% hydrogen, 10% carbon dioxide and 80% nitrogen) with pigment development typically taking 3 days (*P. intermedia*) to 5 days (*P. gingivalis*). *Capnocytophaga ochracea*, *Actinobacillus actinomycetemcomitans* and all anaerobic and facultative clinical isolates were also cultured successfully on FAA and CBA in an anaerobic environment.

3.1.2 LIQUID MEDIUM

All anaerobic and facultative organisms were cultured in FAB before DNA extraction (black-pigmented bacteria, *C. ochracea*, *A. actinomycetemcomitans* and some clinical isolates) or BHI and GLC broths (clinical isolates) and grew successfully. Pigment production was not always observed from *Prevotella* species and *Porphyromonas gingivalis* in liquid media.

3.2 GROWTH AND MAINTENANCE OF AEROBIC AND FACULTATIVE BACTERIAL SPECIES

3.2.1 SOLID MEDIUM

Staphylococcus aureus and all aerobic clinical isolates were maintained on CBA, growth was typically quick with large numbers of colonies seen after 24 h. Some streptococcal species were cultured on TYC to observe extracellular polysaccharide production (see section 3.12.2.3) seen as mucoid type colonies or as colonies surrounded by a gelatinous mass.

3.2.2 LIQUID MEDIUM

Aerobic clinical isolates were grown in BHI broth before DNA extraction or Gram stain and GLC broth for GLC analysis. Growth was successful in both types of broth, but was slower than on solid media, with cultures typically taking 72 h to be considered turbid enough for DNA extraction.

3.3 LONG-TERM STORAGE OF MICROORGANISMS IN FUM

Most microorganisms used during this study (not including those clinical isolates listed as non-viable in table 6.18, appendix 6.18) were stored in FUM (Loesche *et al.*, 1972) at -80°C and were plated directly onto pre-reduced medium from -80°C if required. FUM proved to be an effective storage medium for the majority of laboratory strains and clinical isolates, however a few clinical isolates were not successfully preserved in this manner.

RESULTS

SECTION A: MOLECULAR (DNA) TECHNIQUES

3.4 DNA EXTRACTION

DNA was extracted from Gram-negative and Gram-positive bacteria listed in table 2.1 and Gram-negative and Gram-positive clinical isolates using a phenol-chloroform extraction and the Puregene DNA isolation kit (Gentra Systems Inc.) respectively (sections 2.4.1 and 2.4.2). Both techniques were successful in the majority of occasions, although a range of DNA concentrations were extracted (see section 3.4.1 below). Before a broth culture was subjected to DNA extraction, a Gram stain was performed using sterile techniques to ensure that the Gram profile was as expected; any contamination or ambiguity of result halted extraction from that culture. An agar plate was inoculated with broth culture at the same time and incubated in the appropriate conditions. All growth was carefully checked against historical information; inconsistencies or contaminated cultures lead to extracted DNA being discarded.

3.4.1 QUANTIFICATION OF THE AMOUNT OF DNA

Extracted DNA was observed by electrophoresis in 0.8% agarose gels. The DNA was seen after ethidium bromide staining, as a broad band of high molecular weight. Smearing of the band was due to digestion of the DNA and the RNA was seen as a broad band of low molecular weight in all preparations from Gram-positive bacteria. For RAPD-PCR and sequence analysis, the amount of DNA used was crudely standardised by visually comparing the intensity of 5 μl extracted DNA with 400 ng, 200 ng, 100 ng, 40 ng and 10 ng of uncut λ DNA. In all cases, a greater amount of DNA was obtained from Gram-negative organisms than Gram-positive ones. In excess of 400 ng was routinely seen in 5 μl of extracted DNA from Gram-negative organisms, although as little as 20 ng was seen on occasion. A value of no more than 40 ng was more commonly seen from Gram-positive organisms. Occasionally, no DNA was seen by electrophoresis following extraction with the Puregene DNA isolation kit (Gentra Systems Inc.) from Gram-positive cultures, but in most cases PCR amplification from the extraction was successful. Where PCR amplification did not occur, the DNA extraction was repeated. DNA (2 μl) intended as template for PCR with specific primers was visually compared to uncut λ DNA of the following concentrations; 1000 ng, 400 ng, 200 ng, 100 ng, 40 ng, 10 ng and 1 ng. DNA standards below 10 ng were not visible by this technique. The use of a broad range of standard concentrations facilitated easy approximation of the amount of DNA.

3.4.2 RNA REMOVAL

RNA removal was only necessary for Gram-negative organisms. The addition of 10 mg/ml RNase A was sufficient to remove all traces of RNA from the extracted nucleic acid, demonstrated by the absence of the low molecular weight smear after gel electrophoresis.

3.5 RAPD-PCR

3.5.1 NEGATIVE AND POSITIVE CONTROLS

No amplification products were seen when sterile water was used as a negative control, any contamination with foreign DNA would cause false positive results; in this study all results were due to the addition of template DNA. Once the RAPD-PCR protocol was established, DNA extractions successfully amplified by primer L10 was used as a positive control for all other work.

3.5.2 OPTIMISATION OF RAPD-PCR CONDITIONS

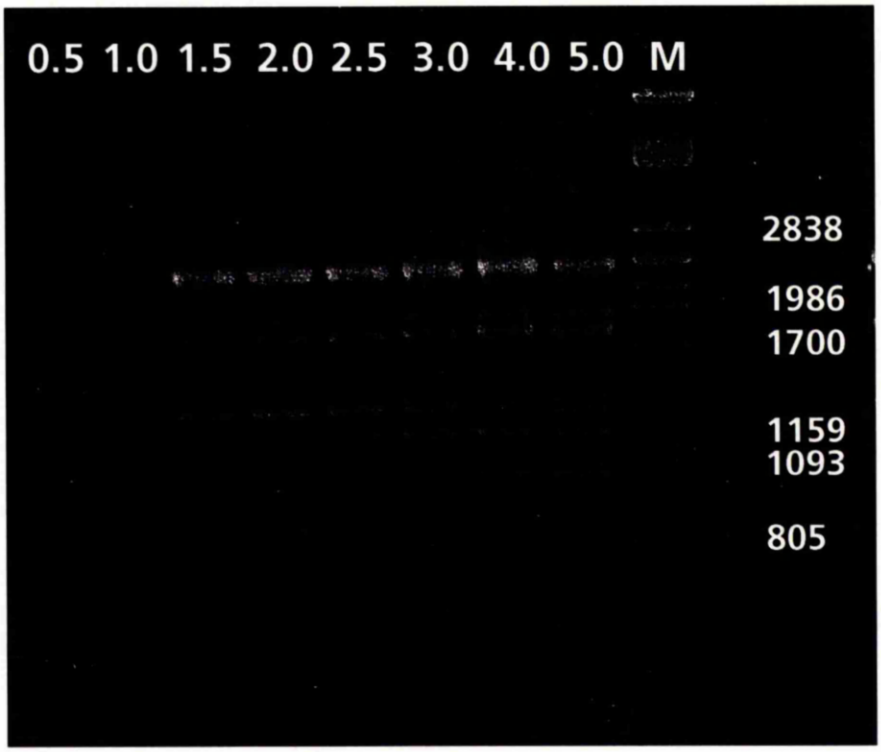
Primer concentration and magnesium concentration was optimised for all four RAPD-PCR primers. For all primers, the number of bands and the intensity with which they are seen increases as the concentration of $MgCl_2$ and primer increases. This is illustrated in figure 3.1 using primer RSP and DNA from *P. intermedia* MH6. Amplification did not occur below 1.0 mM $MgCl_2$ for any random primer but was seen at the lowest primer concentration tested (0.5 μM). Increasing the concentration above an optimum results in an increase in low intensity amplification products. Optimum concentrations of primer and of $MgCl_2$ are tabulated in table 3.1.

Table 3.1 Optimal primer and magnesium concentrations for each random primer used for RAPD-PCR

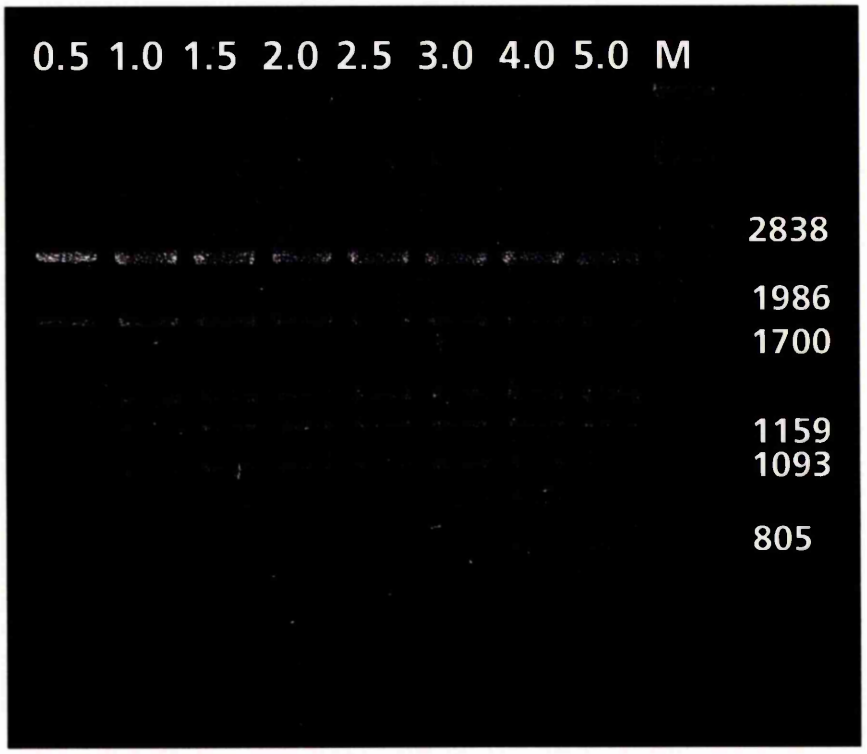
	970-11	L10	RSP	US
Primer concentration (μM)	1.0	2.0	1.5	1.0
Magnesium concentration (mM)	3.0	3.0	3.0	2.0

Figure 3.1 **Effect of increasing magnesium and primer concentrations on RAPD-PCR amplification profile using DNA from *P. intermedia* MH6 and random primer RSP**

(a) Magnesium concentration (0.5 – 0.5 mM)



(b) Primer concentration (0.5 – 0.5 μM)



3.5.3 REPRODUCIBILITY OF RAPD-PCR AMPLIFICATION PROFILES

RAPD-PCR reactions were tested for their reproducibility between reactions by ensuring all reactions were performed on three separate occasions (section 2.5.4). Three (970-11, US, RSP) out of the four random primers failed to amplify target DNA consistently. Only one primer, L10 gave continued amplification for all samples over the course of the study. It became apparent that there were fluctuations in the intensity of amplified products and occasionally, previously amplified bands were not seen. Only those reproducible bands that appeared in all three separate reactions were chosen for statistical analysis and possible species distinction.

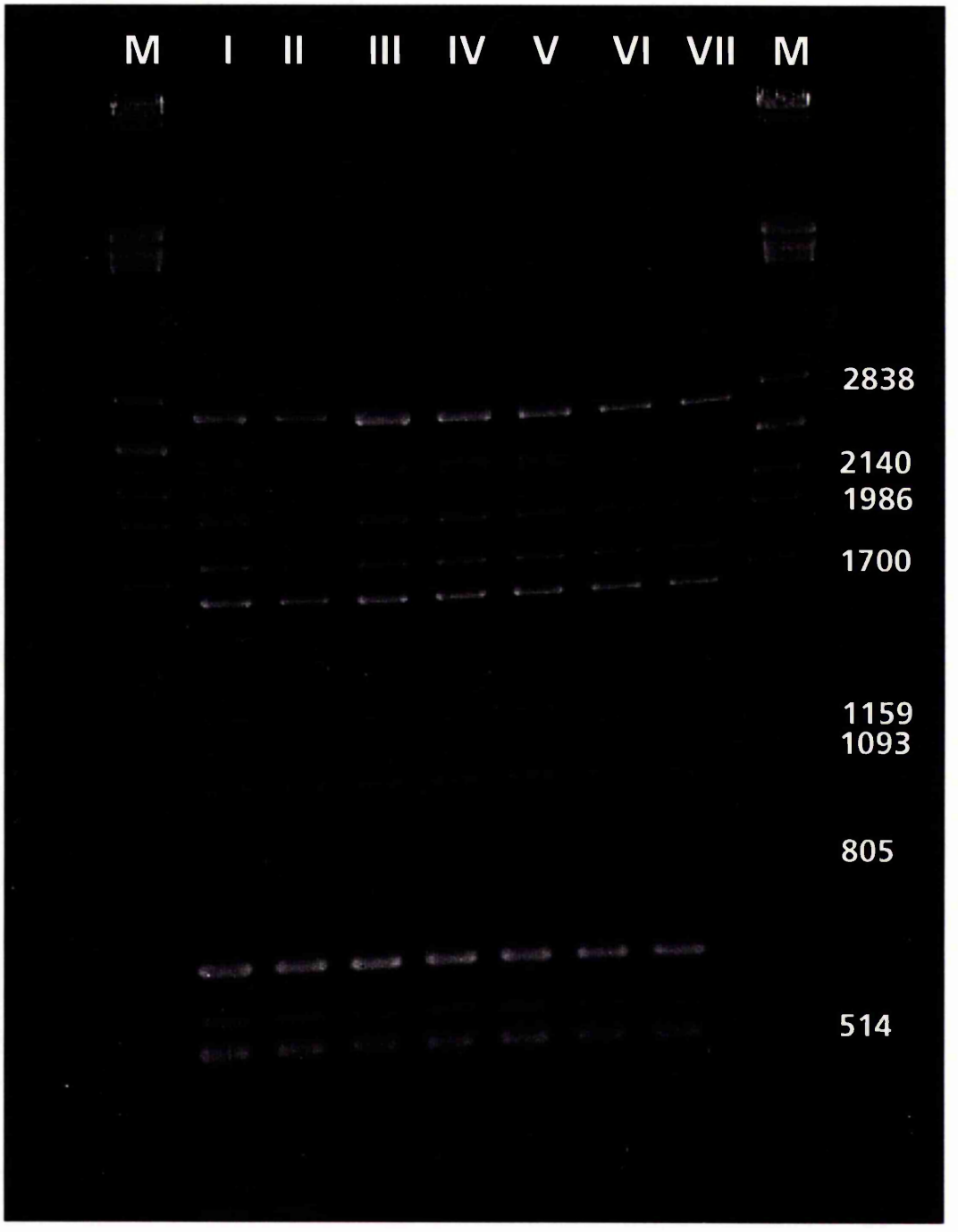
3.5.4 THE EFFECT OF RNA ON RAPD-PCR AMPLIFICATION PROFILES

Three RAPD-PCR analyses were performed as described in section 2.5 using DNA from which RNA had been removed. The amplification patterns seen after electrophoresis was identical for template DNA including RNA and template DNA from which RNA had been removed. The presence of RNA was shown to have no effect on RAPD-PCR results.

3.5.5 STABILITY OF EXTRACTED DNA AND ABILITY TO PRODUCE REPRODUCIBLE AMPLIFICATION PROFILES OVER TIME

As described in section 2.5.6, DNA was extracted monthly over a seven month period and amplified by RAPD-PCR each month over 15 months as illustrated by table 2.3 (materials and methods). Extracted DNA stored in TE produced reproducible RAPD profiles from time of extraction until month 15; thus major bands were identical regardless of the time of DNA extraction and the length of storage time (figure 3.2). Figure 3.2 illustrates DNA extracted from *P. intermedia* MH3 extracted at months 1-7 and amplified with primer L10 at month 15. The DNA extractions illustrated ranged from 8 months (extracted at month 7) to 15 months (extracted month 1) old. Variations in intensity of some bands were seen over the time frame, especially in bands below approximately 514 - 468 bp (this doublet runs as a single band within the λ *Pst* I digest marker) which bears no relation to the age of the DNA extraction. However, the banding patterns are essentially the same indicating DNA stability over time.

Figure 3.2 Effect of age of DNA extraction on RAPD-PCR amplification profiles, tested with DNA from *P. intermedia* MH3 and random primer L10



KEY:

I-VII, month of DNA extraction (see also table 2.3)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

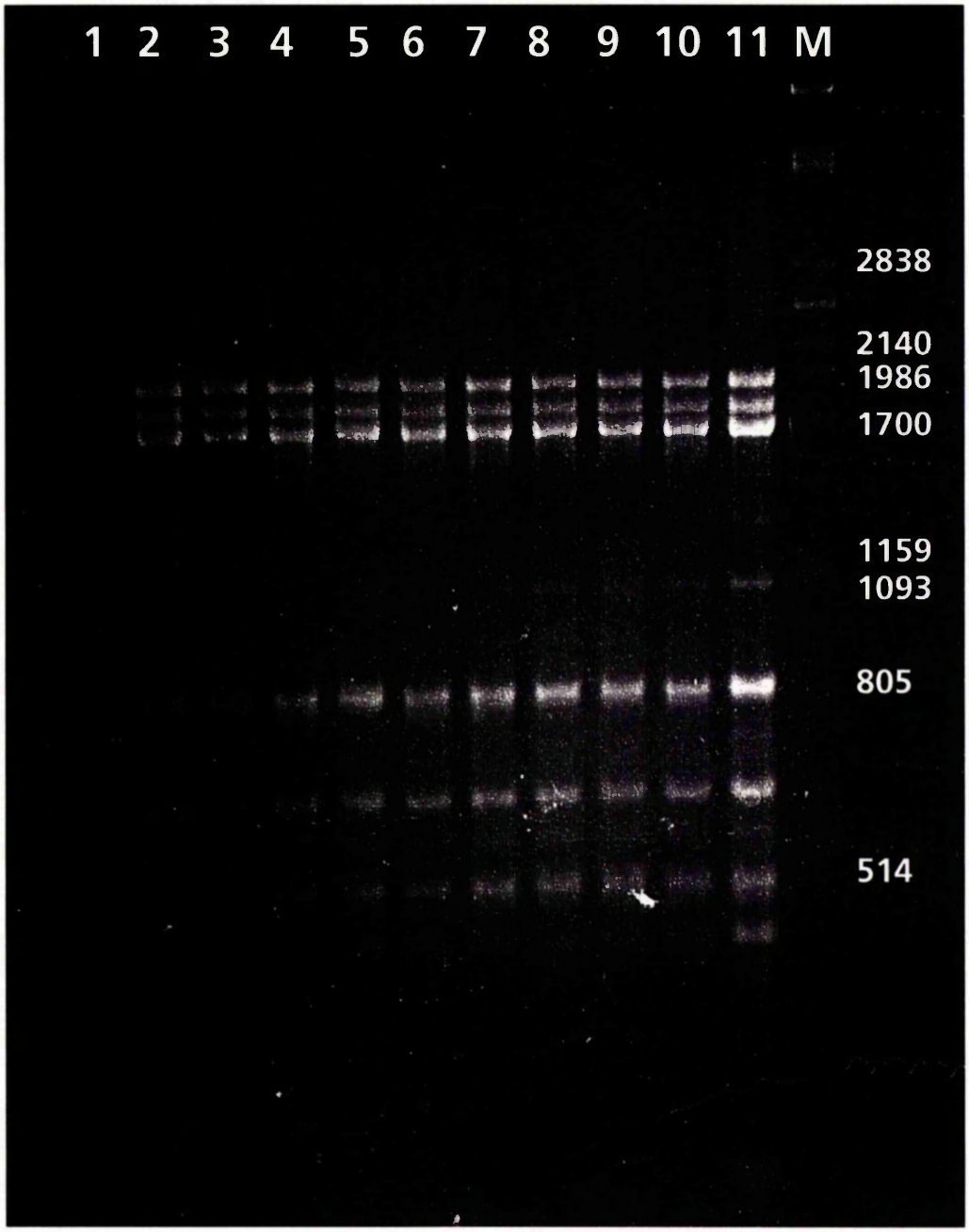
3.5.6 EFFECT OF DNA TEMPLATE CONCENTRATION ON RAPD-PCR AMPLIFICATION PROFILES

The amount of DNA in 1 µl of an extraction from *P. nigrescens* strain LM94 was determined as described in section 2.4.3 and approximately 100 ng was found to be present. This was diluted 1:2, 1:5, 1:10, 1:15, 1:20, 1:25, 1:50, 1:75, 1:100 and 1:200 giving an approximate range of DNA concentrations (per microlitre) as follows: 100 ng, 50 ng, 20 ng, 10 ng, 6.6 ng, 5 ng, 4 ng, 2 ng, 1.3 ng, 1 ng and 0.5 ng (500 pg). These dilutions were examined by electrophoresis as described in section 2.4.3 but concentrations below 1:10 (10 ng) could not be seen.

These 10 dilutions plus undiluted stock (5 µl) were used for RAPD-PCR with primer L10 as described in section 2.5.7. Figure 3.3 demonstrates the result. As the concentration of template (per reaction volume) decreases from 500 ng to 2.5 ng, the intensity of major bands increases. At 500 ng (undiluted stock) there is little amplification of bands below 1700 bp and after 250 ng (1:2; 50ng; lane 2) the same major bands are consistently amplified. A new major band of low molecular weight appears at 50 ng (1:10; 10 ng; lane 4) and is amplified at all lesser concentrations. At concentrations below 1:15 (6.6 ng; 33 ng for RAPD-PCR; lane 5) low intensity bands (around 1093 bp and below 500 bp) are seen, which increase in intensity as the amount of template decreases.

The use of high concentrations of template, in this case 500 ng (undiluted template) resulted in a lower intensity of amplification. At the lowest concentration (2.5 ng; lane 11) a marked increase in new bands was seen. RAPD-PCR was successful at estimated DNA concentrations of 500 ng to 2.5 ng per reaction volume with concentrations in the range of 250 ng to 2.5 ng showing no significant alteration to the banding pattern.

Figure 3.3 Effect of DNA template concentration on RAPD-PCR amplification profiles using a range of concentrations of DNA from *P. nigrescens* LM94 and random primer L10



KEY:

Numbers 1–11, template DNA dilution from stock, value in parentheses represents amount used in PCR reaction. 1, stock DNA (approximately 500 ng); 2, 1:2 dilution (250 ng); 3, 1:3 (100 ng); 4, 1:10 (50 ng); 5, 1:15 (33.5 ng); 6, 1:20 (25 ng); 7, 1:25 (20 ng); 8, 1:50 (10 ng); 9, 1:75 (6.7 ng); 10, 1:100 (5 ng); 11, 1:200 (2.5 ng)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

3.6 RAPD-PCR OF *P. INTERMEDIA* AND *P. NIGRESCENS* DNA

Unique banding patterns were obtained with each of the four random primers for all *P. intermedia* and all *P. nigrescens* strains.

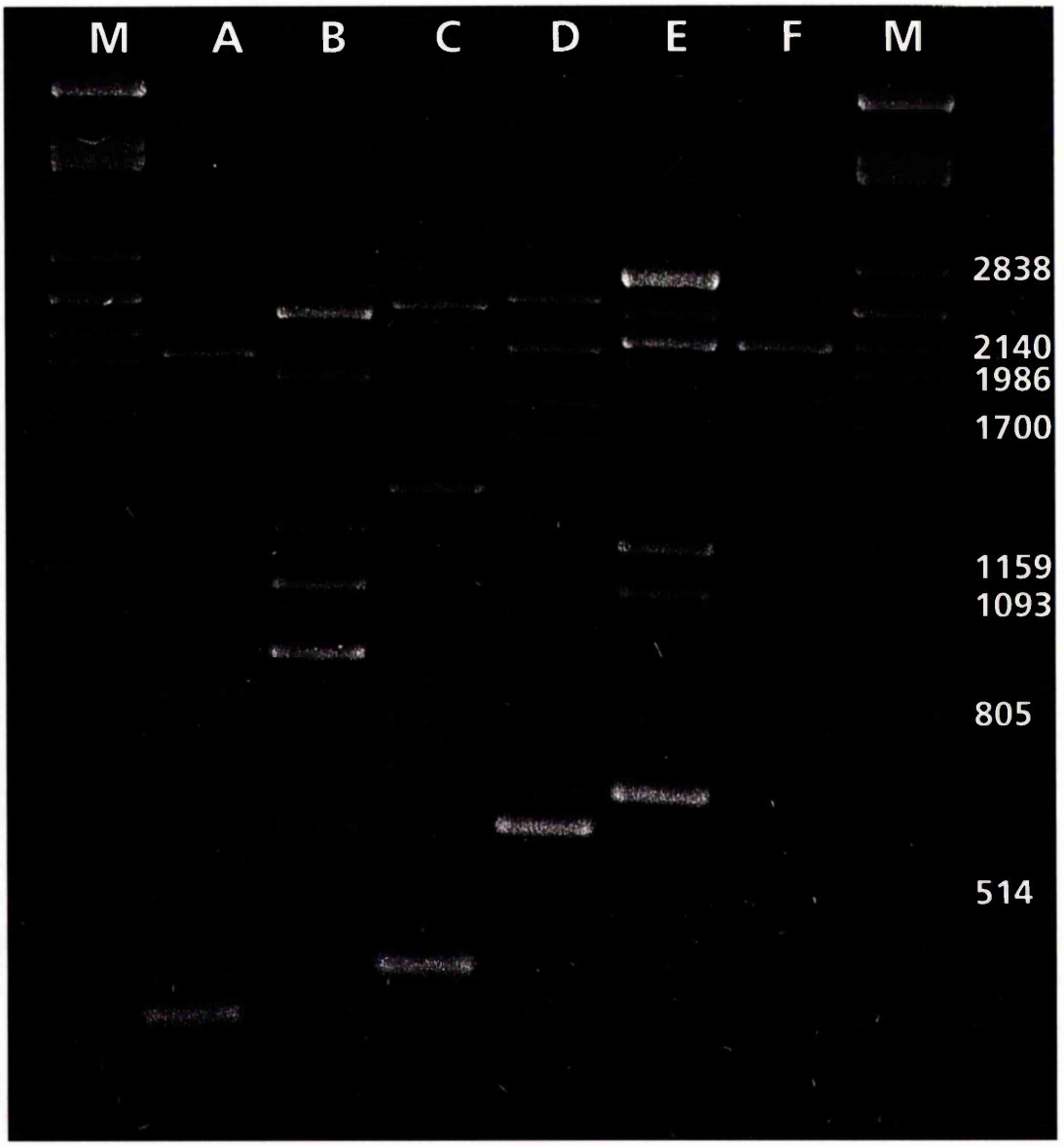
3.6.1 PRIMER, RSP, US AND 970-11

Under the conditions described, the primers RSP, US and 970-11 produced informative banding patterns which demonstrated a large level of inter-species heterogeneity between *P. intermedia* and *P. nigrescens* as well as intra-species heterogeneity between strains of the species. Amplification with primers RSP, US and 970-11 was inconsistent, with an absence of any amplification products seen frequently. On the occasions when amplification was successful, high levels of strain discrimination were seen when amplification with primer 970-11 was performed. Figure 3.4 illustrates the amplification of DNA from a limited number of *P. intermedia* and *P. nigrescens* strains (see section 2.5.8) using primer 970-11. The banding patterns achieved by amplification with primer US showed some intra-species homogeneity between strains of *P. intermedia* (figure 3.5). The same limited number of strains were tested as for primer 970-11 (section 2.5.7), and bands of approximately 1980 bp (band I) and 1160 bp (band III) were seen in 3 out of 4 strains of *P. intermedia* (MH12, MH15 and ATCC 25611). *P. intermedia* MH6, MH12 and ATCC 25611 all shared a band at 1700 bp (band II) and all 4 strains had one at approximately 870 bp (band IV). A smaller band of approximately 310 bp (band V) was amplified from 3 strains (*P. intermedia* MH6, MH12 and MH15). A *P. nigrescens* specific band was seen at approximately 281 bp (band VI). Bands I -VI are labelled in figure 3.5. The inconsistency of successful amplification coupled with the high level of diversity shown by these three primers meant that they were considered unsuitable for species differentiation. See appendix 6.3 for information regarding the absence of RAPD-PCR amplification products.

3.6.2 PRIMER L10

The banding profiles produced by electrophoresis of PCR products from amplification with L10 showed intra-species homogeneity, within both *P. intermedia* and *P. nigrescens*, highlighting the genetic similarities between strains of the same species. These characteristic amplification patterns are illustrated in figure 3.6.

Figure 3.4 RAPD-PCR amplification profiles of DNA from 4 *P. intermedia* and 2 *P. nigrescens* strains amplified with random primer 970-11



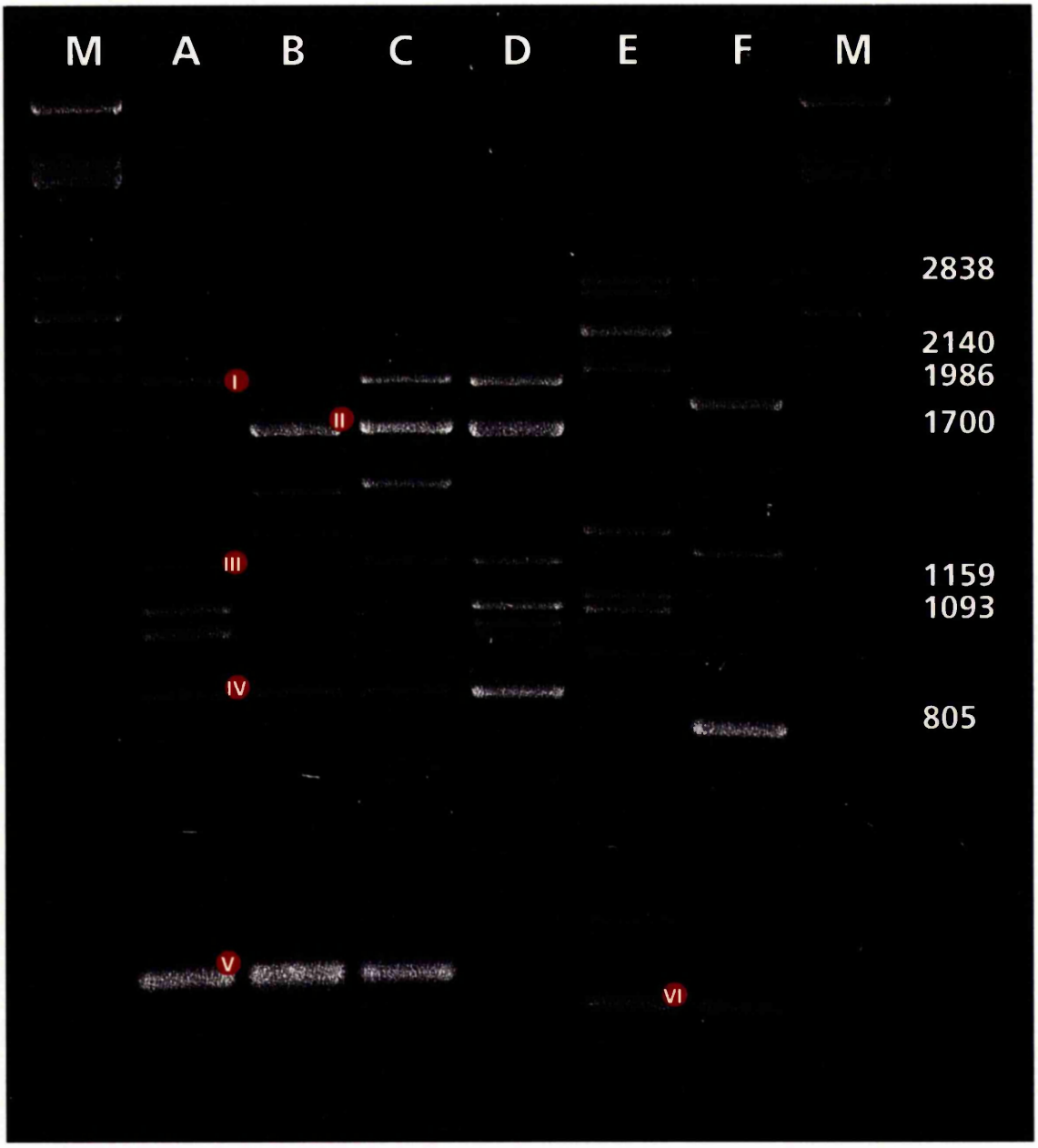
KEY:

Lanes A-D, *P. intermedia* strains. A, *P. intermedia* MH15; B, *P. intermedia* MH6; C, *P. intermedia* MH12; D, *P. intermedia* ATCC 25611

Lanes E-F, *P. nigrescens* strains. E, *P. nigrescens* LM94; F, *P. nigrescens* MH4

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Figure 3.5 RAPD-PCR amplification profiles of DNA from 4 *P. intermedia* and 2 *P. nigrescens* strains amplified with random primer US



KEY:

Lanes A-D, *P. intermedia* strains. A, *P. intermedia* MH15; B, *P. intermedia* MH6; C, *P. intermedia* MH12; D, *P. intermedia* ATCC 25611

Lanes E-F, *P. nigrescens* strains. E, *P. nigrescens* LM94; F, *P. nigrescens* MH4

I-VI, bands referred to in text (section 3.6.1)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Strains of *P. intermedia* share bands of approximately 1500 bp (labelled band II) and 600 bp (labelled band IV) and strains of *P. nigrescens* have bands at approximately 1900 bp (labelled band I) and 805 bp (labelled band III). These four bands were seen to be consistently amplified and displayed as bands of high intensity. A band common to strains of *P. intermedia* and *P. nigrescens* was seen at approximately 1750 bp (band V). These bands are labelled in figure 3.6.

3.6.3 STATISTICAL ANALYSIS - RAPD-PCR OF *P. INTERMEDIA* AND *P. NIGRESCENS* DNA

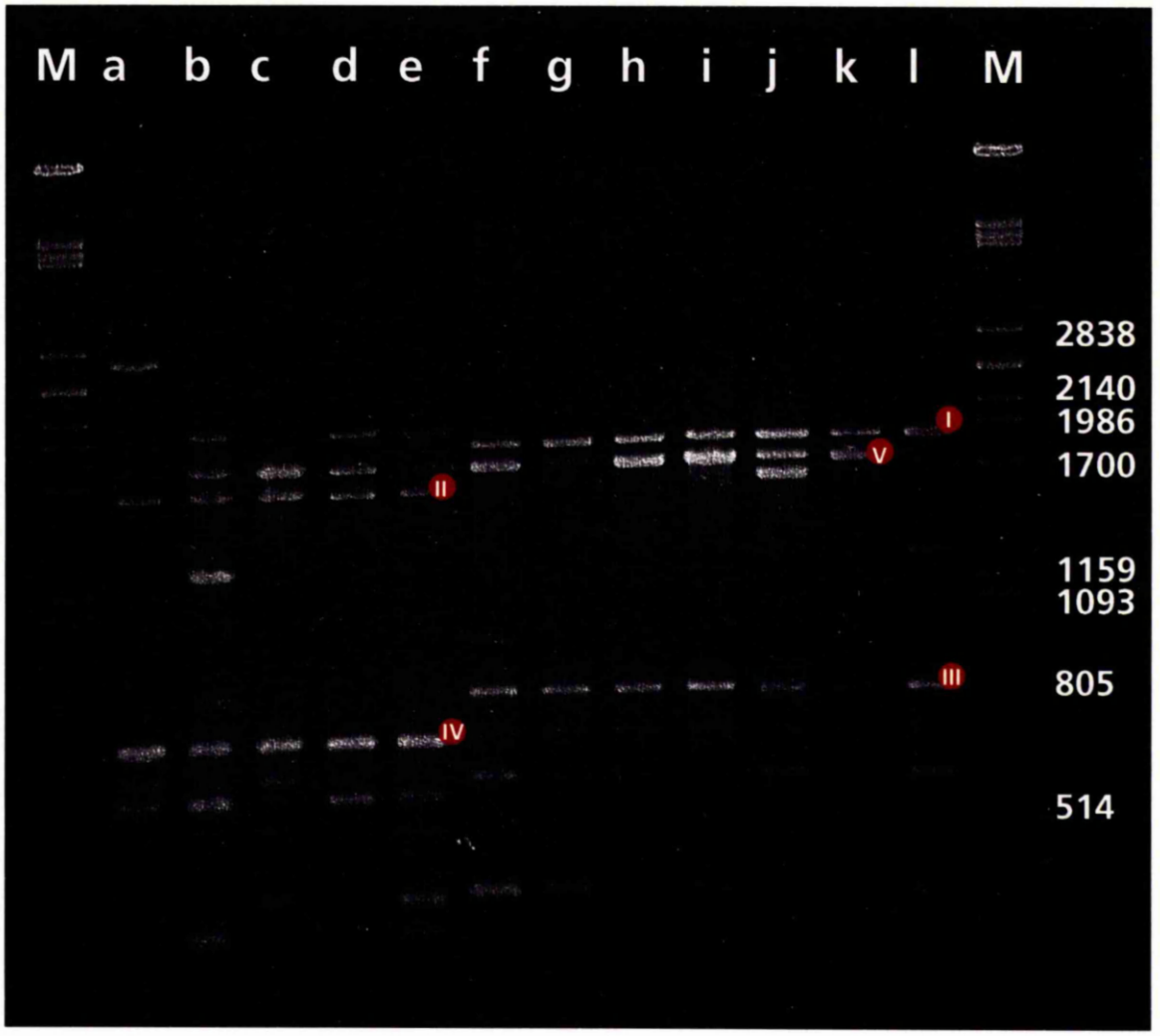
After electrophoresis, the approximate sizes (bp) of the 15 reproducible bands to be scored were determined (see appendix 6.4, table 6.1 A, figure 6.1). The reproducible bands were scored visually as 1 (present) or 0 (absent) for each strain of *P. intermedia* and *P. nigrescens* and a binary matrix constructed. As described in the materials and methods section 2.5.10, a dendrogram was constructed, using average linkage between groups (also called UPGMA or unweighted pair-group method using arithmetic averages) and the simple matching coefficient.

The dendrogram (I) is shown in figure 3.7. Statistical analysis of the RAPD-PCR results delineates 2 distinct clusters: cluster I which groups all *P. nigrescens* strains together and independently from *P. intermedia* strains (cluster II). The 2 clusters meet at approximately 50% similarity.

Strains of *P. nigrescens* form a tighter cluster with strains displaying a maximum of 93% and a minimum of 78% similarity. Cluster II shows less similarity amongst *P. intermedia* strains compared to *P. nigrescens* with a maximum of 87% and a minimum similarity of 65%. The 2 *P. nigrescens* type strains ATCC 25261 and ATCC 33563 are quite separated; *P. nigrescens* ATCC 25261 clusters with *P. nigrescens* MH4 (93% similarity; 1 band difference) whilst *P. nigrescens* ATCC 33563 is alone at the base of the cluster and does not group tightly with any other strains. Amongst *P. intermedia* strains, *P. intermedia* MH6 is alone at the base of the cluster joining the other 4 strains at a value of 65% similarity. The *P. intermedia* type strain ATCC 25611 is most similar to *P. intermedia* MH15 (80% similarity; 3 band differences).

Figure 3.6 RAPD-PCR amplification profiles of DNA from 5 *P. intermedia* and 7 *P. nigrescens* strains amplified with random primer L10

Species-specific bands I-IV and shared band V are highlighted



KEY:

Lanes a-e, *P. intermedia* strains. a, *P. intermedia* MH3; b, *P. intermedia* MH6; c, *P. intermedia* MH12; d, *P. intermedia* MH15; e, *P. intermedia* ATCC 25611

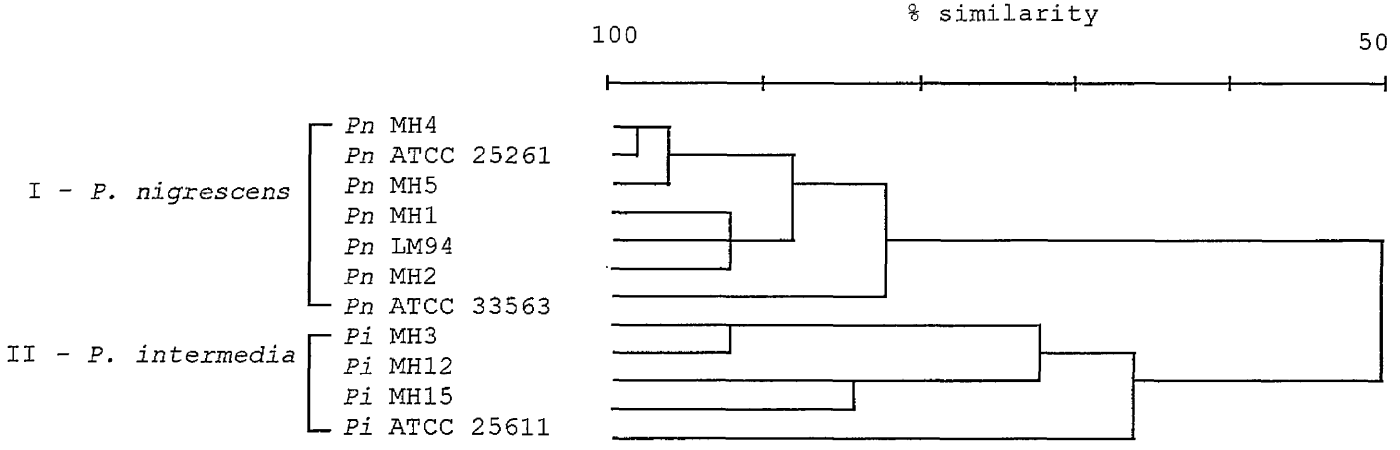
Lanes f-l, *P. nigrescens* strains. f, *P. nigrescens* MH1; g, *P. nigrescens* MH2; h, *P. nigrescens* MH4; i, *P. nigrescens* MH5; j, *P. nigrescens* LM94; k, *P. nigrescens* ATCC 25261; l, *P. nigrescens* ATCC 33563

I-IV (circle), species-specific bands referred to in text; V, shared band (section 3.6.2)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Figure 3.7 **Dendrogram I. Cluster analysis of RAPD-PCR banding patterns of *P. intermedia* and *P. nigrescens* strains using UPGMA and the simple matching coefficient**

Horizontal axis displays percentage similarity between RAPD-PCR profiles.



KEY:
Pi, P. intermedia; Pn, P. nigrescens

3.7 RAPD-PCR OF GRAM-NEGATIVE BLACK-PIGMENTING CLINICAL ISOLATES DNA WITH PRIMER L10

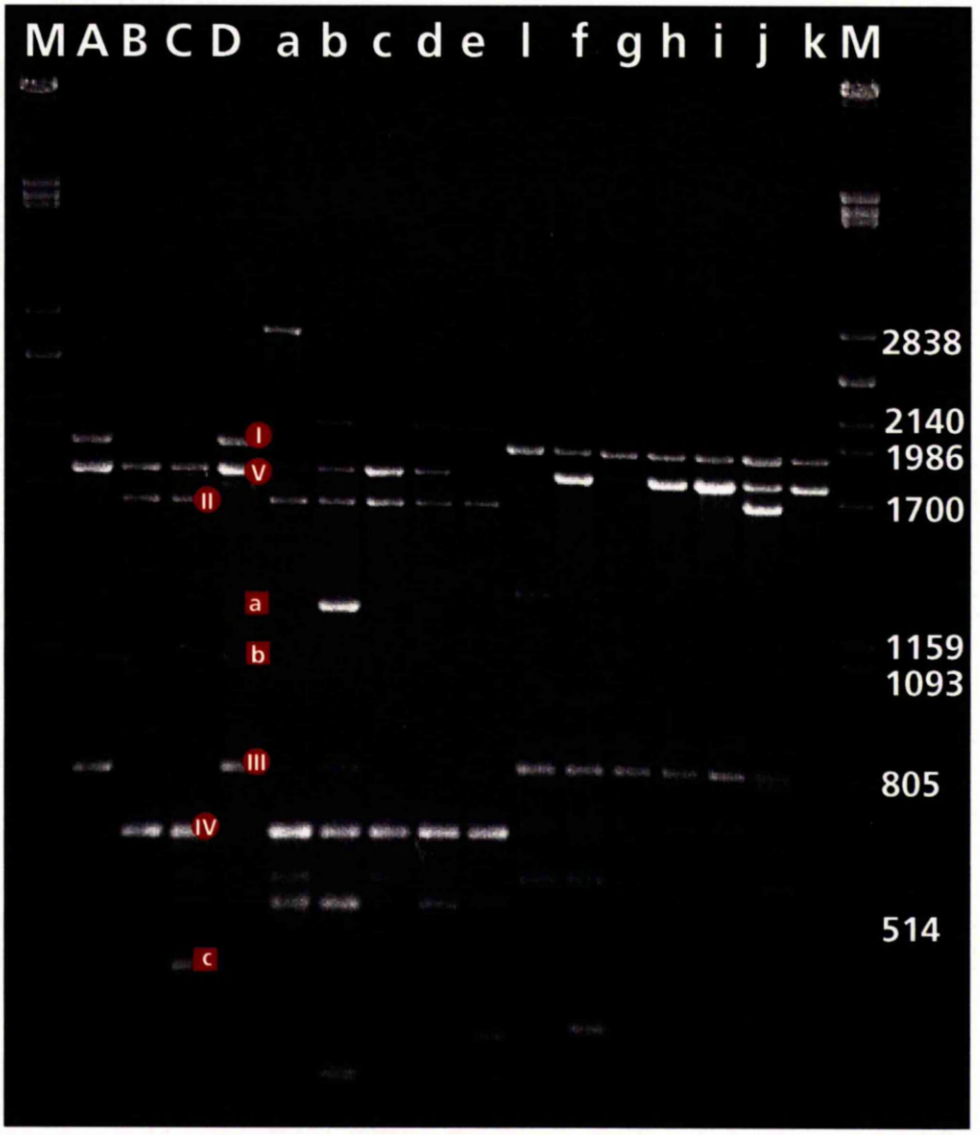
Primer L10 was used to group the 4 black-pigmenting clinical isolates (A-D) identified as *P. intermedia* into either *P. intermedia* or *P nigrescens*. Visual analysis (figure 3.8) showed that the occurrence of bands I-IV facilitated this. By eye, isolates A and D have bands I and II in common with *P. nigrescens* isolates, whilst isolates B and C have bands II and IV like *P. intermedia* isolates. A band common to strains of *P. intermedia* and *P. nigrescens* was seen at approximately 1750 bp (band V). These four bands were seen to be consistently amplified and of high intensity.

3.7.1 STATISTICAL ANALYSIS - GRAM-NEGATIVE BLACK-PIGMENTING CLINICAL ISOLATES

After electrophoresis, the approximate sizes (bp) of the 18 reproducible bands to be scored were determined (see appendix 6.4, table 6.1 B). The species-specific bands were approximately 1900 bp (I), 1650 bp (II), 805 bp (III) and 600 bp (IV) in size and band V approximately 1750 bp. Of these 18 bands, 15 were shared with known *P. intermedia* and *P. nigrescens* strains and 3 (approximately 1180, 1050 and 480 bp) were only found in the profiles of the clinical isolates, these are labelled a-c in figure 3.8. Other faint amplification products were seen for isolates A-D, which were not consistently amplified. A new binary matrix was constructed and a dendrogram (II) produced which is shown in figure 3.9. Two major clusters are seen as previously described for these species (section 3.6.3, figure 3.7), cluster I containing *P. nigrescens* and cluster II containing *P. intermedia.* Isolates A and D fall within cluster I (*P. nigrescens*) and isolates B and C fall within cluster II (*P. intermedia*) confirming the groupings made by visual comparison. The major point to note is that isolates A and D within cluster I have a similarity of 94.4% to each other, and a value of 70% similarity compared to the other *P. nigrescens* strains. This is equivalent to the minimum level of similarity seen with *P. intermedia* MH2 and other strains. Isolate C is most similar to *P. intermedia* MH15 (88%), isolate B joins the cluster containing isolate C at a value of 77%. There are no major differences in the way strains cluster, however all the similarity values have increased slightly with the inclusion of additional bands giving a similarity value between the 2 clusters of 57%.

Figure 3.8 RAPD-PCR amplification profiles of DNA from 5 *P. intermedia*, 7 *P. nigrescens* strains and 4 clinical isolates identified only as *P. intermedia* amplified with random primer L10

Species-specific bands I-IV are highlighted

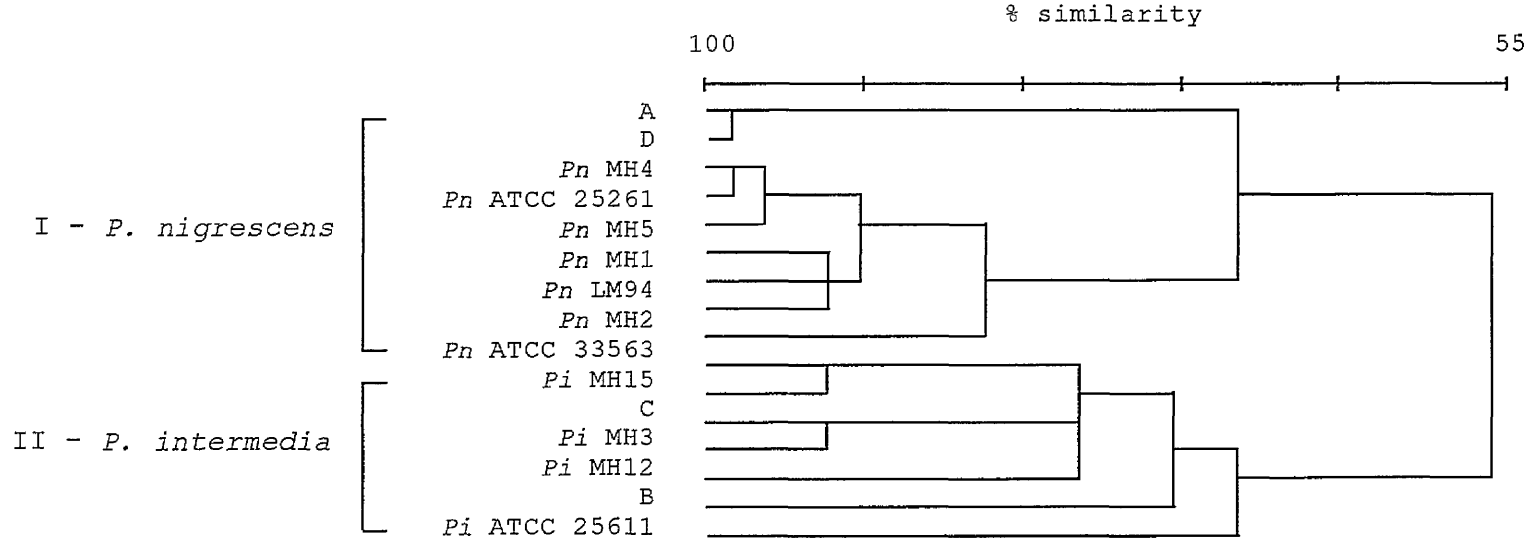


KEY:

Lanes A-D, PLS clinical isolates called A-D; Lanes a-e, *P. intermedia* strains. a, *P. intermedia* MH3; b, *P. intermedia* MH6; c, *P. intermedia* MH12; d, *P. intermedia* MH15; e, *P. intermedia* ATCC 25611; Lanes f-l, *P. nigrescens* strains. f, *P. nigrescens* MH1; g, *P. nigrescens* MH2; h, *P. nigrescens* MH4; i, *P. nigrescens* MH5; j, *P. nigrescens* LM94; k, *P. nigrescens* ATCC 25261; l, *P. nigrescens* ATCC 33563; I-IV (circle), species-specific bands referred to in text; V, shared band (section 3.6.2); a-c (square), new bands scored (section 3.7.1); M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Figure 3.9 Dendrogram II. Cluster analysis of RAPD-PCR banding patterns of *P. intermedia* and *P. nigrescens* strains using UPGMA and the simple matching coefficient

Horizontal axis displays percentage similarity between RAPD-PCR profiles.



KEY:

Pi, P. intermedia; Pn, P. nigrescens; A, PLS isolate **1** *2B*; B, PLS isolate **2** *3A*; C, PLS isolate **5** *1D*; D, PLS isolate **3** *2A*

3.8 RAPD-PCR OF PINLO DNA

RAPD-PCR using primer L10 and DNA extracted from PINLOs suggests homogeneity amongst these strains (figure 3.10). Although the majority of amplified fragments are shared between the 3 PINLOs, strain A391 displays 3 bands (labelled a-c in figure 3.10), of approximately 2140, 1750 and 600 bp (the band at 600 bp represents band IV and that at 1750 bp band V, see section 3.6.2) not seen in amplification profiles of the HST 1156 and HST 2160. In addition, slight variations in the intensities of shared bands can be seen.

3.8.1 RAPD-PCR OF PINLOS COMPARED TO *P. INTERMEDIA, P. CORPORIS, P. NIGRESCENS* AND *P. PALLENS*

Extracted DNA from *P. pallens* NCTC 130Y2 and AHN 9423 and *P. corporis* ATCC 33547, A363 and A350 was amplified by RAPD-PCR with primer L10 and the banding patterns were compared to those obtained by amplification of PINLOs, *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 33563 and ATCC 25261. Figure 3.11 compares the banding patterns obtained for the *Prevotella* species with those of the 3 PINLOs. It illustrates clearly that the PINLOs do not produce amplification products similar to related species. Further comparisons are required to assess the occurrence of a PINLO specific RAPD-PCR band but the amplification products are quite characteristic when compared to other species.

In the same way as *P. intermedia*, *P. nigrescens* and PINLOs have potential species-specific bands or characteristic profiles, amplification with L10 reveals that the 2 *P. pallens* strains and the 3 *P. corporis* strains also have bands and profiles which could be unique to the species.

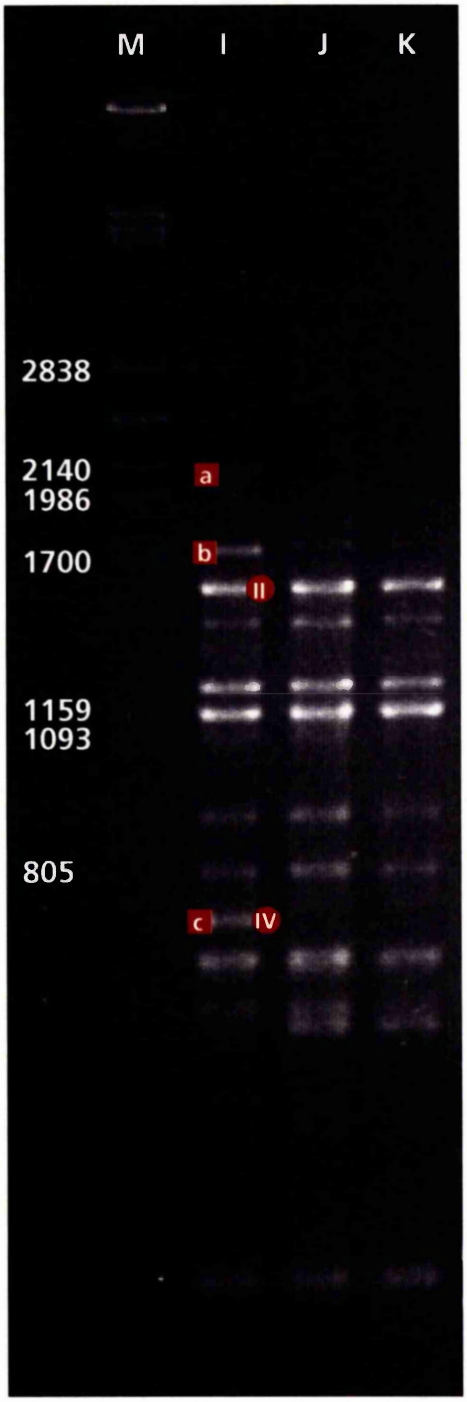
3.8.2 STATISTICAL ANALYSIS - RAPD-PCR OF DNA FROM PINLOS COMPARED TO *P. CORPORIS, P. PALLENS, P. INTERMEDIA* AND *P. NIGRESCENS*

After electrophoresis, the approximate sizes (bp) of the 32 reproducible bands to be scored was determined (see appendix 6.4, table 6.1 C). Of these 32 bands, 8 were shared with *P. intermedia* ATCC 25611 and / or *P. nigrescens* ATCC 33563 and ATCC 25261 and 5 were unique to *P. intermedia* and / or *P. nigrescens*. The remaining 19 were only found in the profiles of PINLOs, *P. corporis* or *P. pallens*. Only 3 of these 19 bands were unique to one or more of the PINLOs (approximately 2140, 1500 and 780 bp), 7 bands (approximately 1450, 1330, 1090, 900, 590 and 2 less than 350 bp) were unique to at least one of the *P. pallens* strains and 3 bands (approximately 500 bp, and 2 less than 350 bp) were unique to

P. corporis. The remaining 6 bands were shared between 2 or more species; 2 between *P. corporis* and *P. pallens* (approximately 1600 and 930 bp), 1 band of approximately 1159 bp was shared between *P. pallens* and PINLOs, 1 band of approximately 1200 bp was shared between *P. corporis* and PINLOs and 2 bands (1 of approximately 495 bp and 1 of less than 350 bp) were shared by at least one strain of each of the 3 species.

A binary matrix containing this information was used to produced the dendrogram (III) displayed in figure 3.12. Five clusters are seen which correspond to the 5 species tested. The PINLOs cluster together and separately from all other species with strains HST 1156 and HST 2160 displaying 100% similarity and A391 90% similarity to the other 2. The least similarity is seen between PINLOs and *P. pallens* confirming the existence of other PINLO strains which cannot be classified as *P. pallens*. The dendrogram displays heterogeneity amongst members of *Prevotella* species and confirms the observation that PINLOs have a low level of heterogeneity amongst strains and that they are distinct from related species. PINLOs, *P. corporis*, *P. intermedia* and *P. nigrescens* strains cluster with 62% similarity, the 2 *P. pallens* strains meet at only 68% similarity and cluster with the other species at 55%. As noted previously consideration of additional bands and species increases the similarity levels between *P. intermedia* and *P. nigrescens* which meet here at a value of 62%.

Figure 3.10 RAPD-PCR amplification profiles of DNA from 3 PINLO strains amplified with random primer L10



KEY:

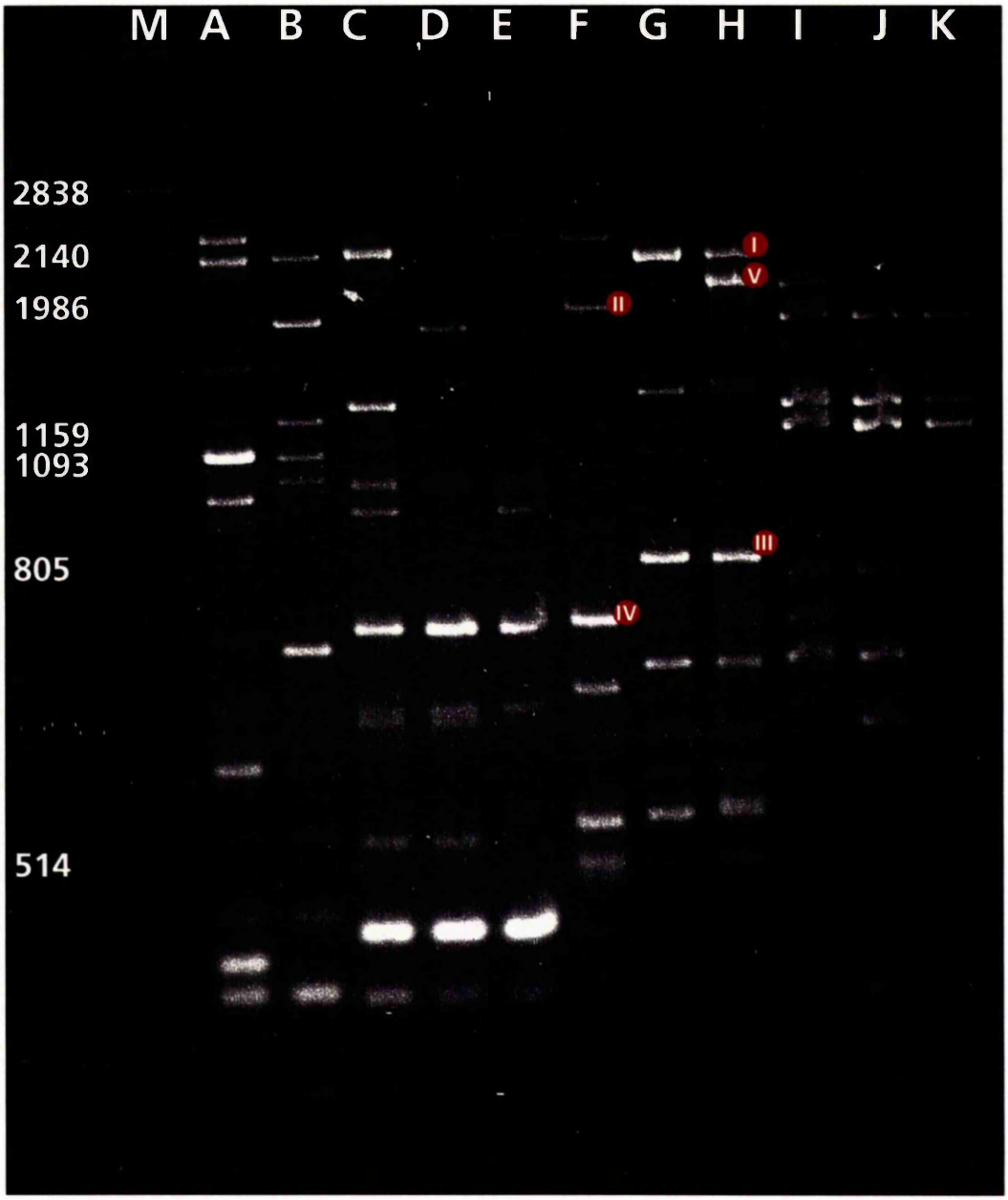
Lanes I-K, PINLO strains. I, PINLO A391; J, PINLO HSS 1156; K, PINLO HSS 2160

II and IV (circle), *P. intermedia*-specific bands referred to in text (section 3.6.2)

a-c (square) bands unique to PINLO A391 (section 3.8)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Figure 3.11 RAPD-PCR amplification profiles of DNA from 3 PINLO strains compared to those from other *Prevotella* species amplified with L10



KEY:

Lanes A-B, *P. pallens*., A, *P. pallens* NCTC 130Y2; B, *P. pallens* AHN 9423; Lanes C-E, *P. corporis*, C. *P. corporis* ATCC 33547; D, *P. corporis* A363; E, *P. corporis* A350; F, *P. intermedia* ATCC 25611; G, *P. nigrescens* ATCC 25261; H, *P. nigrescens* ATCC 33563

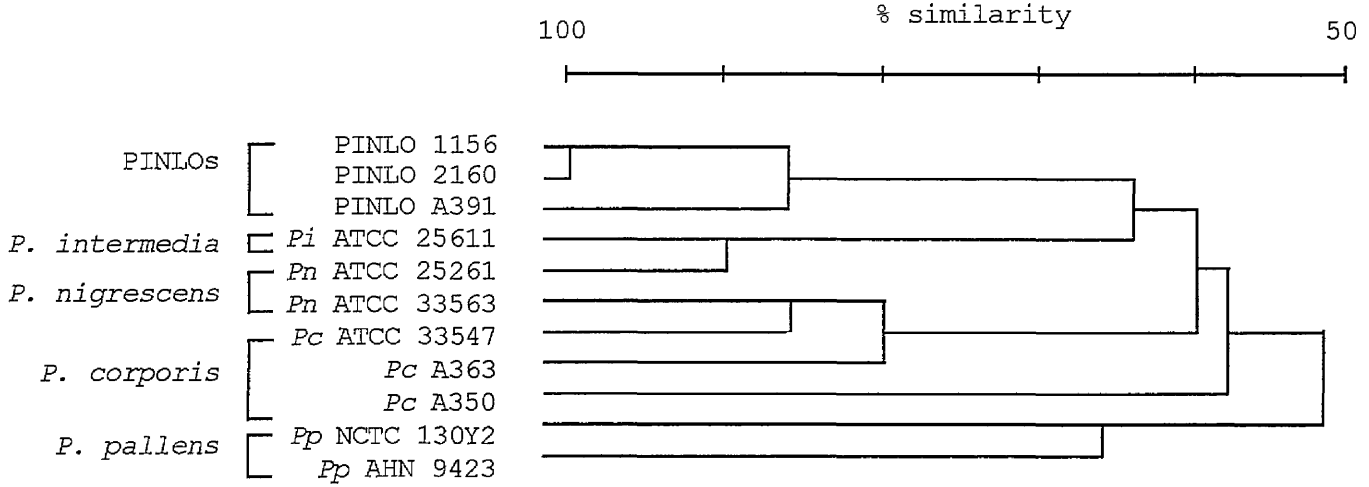
Lanes I-K, PINLO strains, I, PINLO A391; J, PINLO HSS 1156; K, PINLO HSS 2160

I-V (circle), *P. intermedia*-specific bands referred to in text (section 3.6.2)

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

Figure 3.12 Dendrogram III. Cluster analysis of RAPD-PCR banding patterns of PINLO strains compared to *P. corporis*, *P. intermedia*, *P. nigrescens* and *P. pallens* strains, using UPGMA and the simple matching coefficient

Horizontal axis displays percentage similarity between RAPD-PCR profiles.



KEY:
Pc, P. corporis; Pi, P. intermedia; Pn, P. nigrescens; Pp, P. pallens

3.9 PARTIAL 16S rDNA SEQUENCE ANALYSIS

3.9.1 *P. INTERMEDIA*, *P. NIGRESCENS* AND CLINICAL ISOLATES

Approximately 500 bp of the 16S rRNA gene from known *P. intermedia*, *P. nigrescens* and PINLO strains were sequenced and analysed as described in section 2.6. The sequences are displayed in appendix 6.7. The partial 16S rDNA sequences of known *P. intermedia* and *P. nigrescens* strains and clinical isolates A-D, were aligned with entire 16S rRNA gene sequences of *P. intermedia* and *P. nigrescens* found within the EMBL database (see appendix 6.8 for an example of the manipulation of sequence information as described in section 2.6.8). LINEUP was used to remove areas of insertions or deletions and after the edges of the sequences were trimmed to leave only certain sections of sequence, the distances (DISTANCES) were calculated between sequences 388 bases in length. A distance matrix was produced (appendix 6.10.1) which was transferred into SPSS for Windows version 6.1 and a dendrogram plotted. Dendrogram IV (figure 3.13) shows the relationships between stains of *P. intermedia*, *P. nigrescens* and the 4 clinical isolates, 2 clusters are seen corresponding to *P. nigrescens* (cluster A) and *P. intermedia* (cluster B). This analysis confirms that isolates A and D have a high similarity to *P. nigrescens* and isolates B and C to *P. intermedia*.

The alignment that was used to calculate distances is shown in appendix 6.9.1. The majority of variability is constant between strains of a species, for example at position 72, all *P. nigrescens* strains have a T, whilst *P. intermedia* have a C. This type of difference is seen in 24 positions (labelled a-z in appendix 6.9.1). Several of these contain international base codes, however position 306 (w) is slightly more complicated. At position 306 one *P. nigrescens* includes a Y which equals a C or a T , all others have a C, *P. intermedia* (**5** *1D*; C) has a K which equals a G or a T, all other *P. intermedia* strains show a T, except *P. intermedia* (**2** *3A*; B) which has a G. The most variability between species is seen in the 100 -150 base region (16 differences; e-t).

Some single changes or ambiguities within a species are also seen (shown in bold in the alignment in appendix 6.9.1. Position numbers refer to this alignment). *P. intermedia* MH3 has a T at position 18 whilst all other strains of *P. intermedia* plus *P. nigrescens* have a C. At position 26, *P. nigrescens* ATCC 25261 and ATCC 33563 have a T where all others have a C. *P. nigrescens* NCTC 9336 displays a C and an A instead of an A and a G at positions 139 and 142 respectively. *P. intermedia* (**5** *1D*; C) and *P. intermedia* ATCC 25611 have a C at position 380 where all other strains have a T. At positions 175, 203,

379 and 386 there is no clear consensus. The largest area with no sequence differences is between 204 and 305.

Within the *P. nigrescens* cluster (cluster A) the highest similarity levels (determined by lowest distance values) are between PLS isolate A (**1** *2B*) and PLS isolate D (**3** *2A*) and PLS isolate A (**1** *2B*) and *P. nigrescens* MH2. Within the *P. intermedia* cluster (cluster B) the lowest distance figure is seen (excluding the 2 EMBL entries for *P. intermedia* ATCC 25611) between *P. intermedia* MH6 and *P. intermedia* MH12 followed by *P. intermedia* ATCC 25611, *P. intermedia* MH12 and *P. intermedia* MH15. Approximately 91% similarity is seen between the partial 16S rDNA sequences of *P. intermedia* and *P. nigrescens* and 98% within each species.

3.9.2 PINLO STRAINS

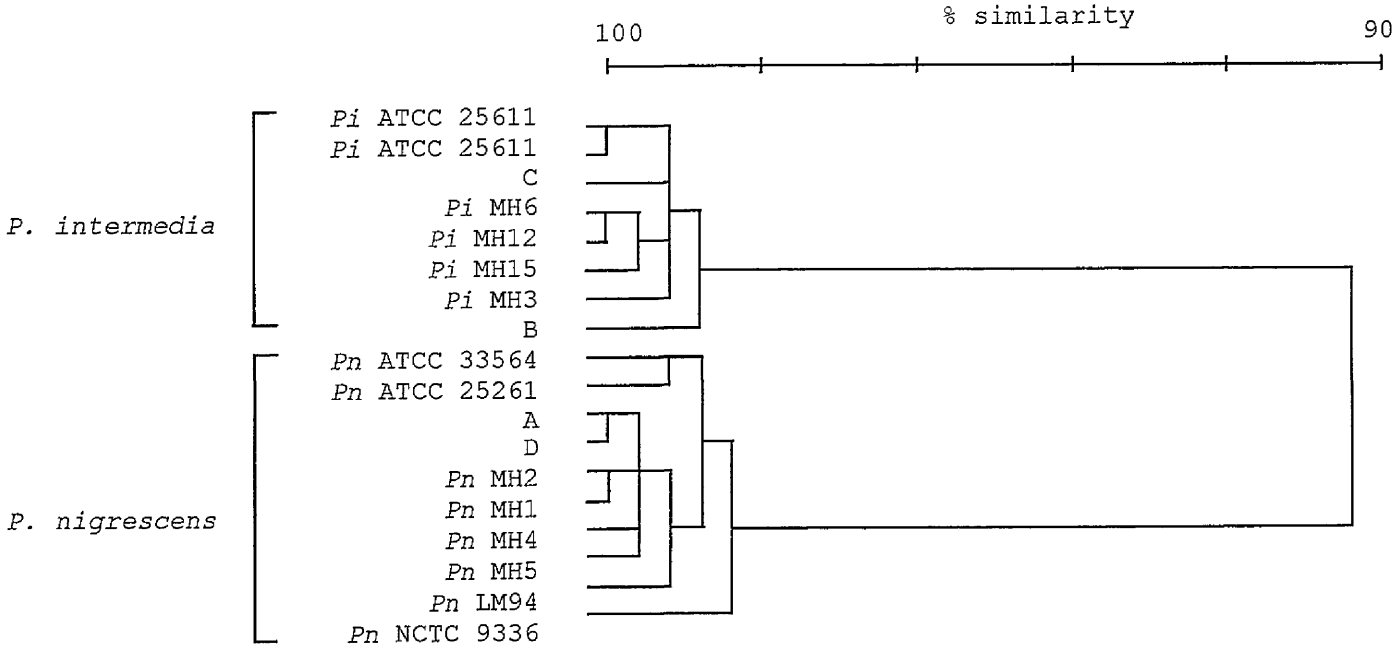
In addition to *P. intermedia* and *P. nigrescens* sequences, PINLO sequences were aligned with *P. corporis*, *P. denticola* and *P. pallens* sequences contained within the EMBL database (see appendix 6.8 for an example of the manipulation of sequence information as described in section 2.6.8). LINEUP was used to remove areas of insertions or deletions and after the edges of the sequences were trimmed, the distances (DISTANCES) were calculated between sequences 441 bases in length. A distance matrix was produced (appendix 6.8.3.4) which was transferred into SPSS for Windows version 6.1 and a dendrogram plotted using the UPGMA method. No coefficient was used due to the method with which data was entered. Dendrogram V (figure 3.14) shows that partial 16S rDNA sequence analysis clusters the PINLO strains together and separately from other *Prevotella* species. The sequences of HST 1156 and HST 2160 are identical over the 441 bases that were aligned. A391 differed from HST 1156 and HST 2160 in 5 positions (435, 436, 438, 440 and 441). This is the very end of the partial sequence of A391.

The aligned sequences used to calculate distances are shown in appendix 6.9.2. In these aligned partial sequences, there are 85 positions with no consensus.

The dendrogram shows approximately 98% similarity within PINLO and confirms the sequence identity of HST 1156 and HST 2160. A close relationship to *P. corporis* (approximately 94% similarity) rather than the other *Prevotella* species is also suggested.

Figure 3.13 Dendrogram IV. Visual representation of distances between partial 16S rDNA sequences for strains of *P. intermedia* and *P. nigrescens*, including clinical isolates A-D

Horizontal axis displays approximate percentage similarity between partial 16S rDNA sequences, clustered using the UPGMA method.

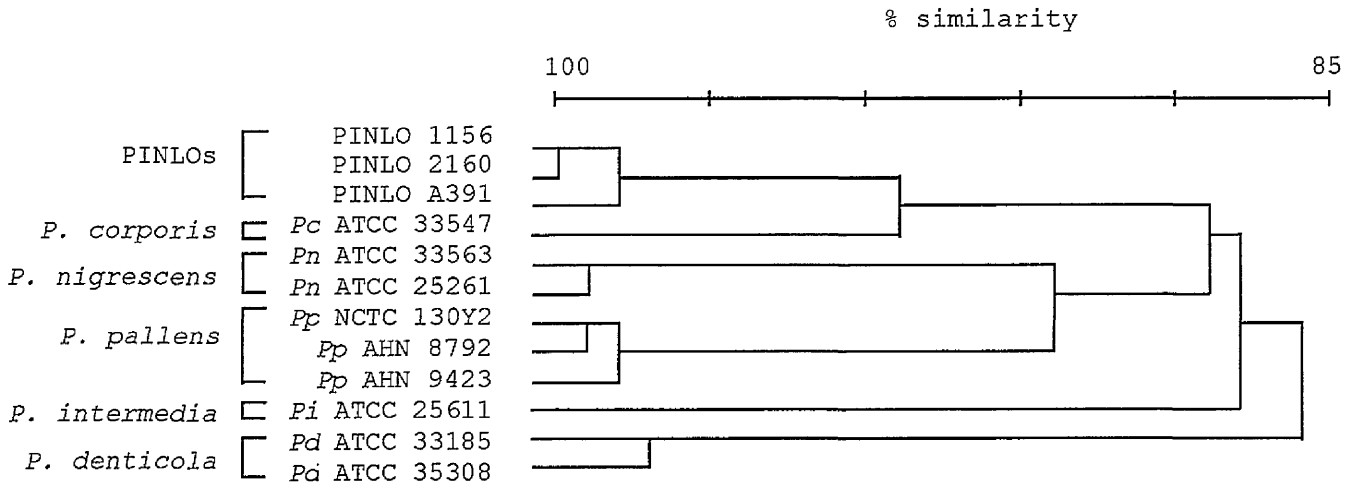


KEY: *Pi, P. intermedia; Pn, P. nigrescens;* A, PLS isolate **1** *2B*; B, PLS isolate **2** *3A*; C, PLS isolate **5** *1D*; D, PLS isolate **3** *2A*

See appendicies 6.7.1-6.7.3 for partial 16S rDNA sequences, appendix 6.6 for EMBL information regarding *Prevotella* type strains and appendix 6.10.1 for sequence distance matrix.

Figure 3.14 Dendrogram V. Visual representation of distances between partial 16S rDNA sequences of PINLO strains and other *Prevotella* species

Horizontal axis displays approximate percentage similarity between partial 16S rDNA sequences, clustered using the UPGMA method.



KEY:

Pc, P. corporis; Pd, P. denticola; Pi, P. intermedia; Pn, P. nigrescens; Pp, P. pallens

See appendix 6.7.4 for PINLO partial 16S rDNA sequences, appendix 6.6 for EMBL information regarding all *Prevotella* species and appendix 6.8.3.4 for sequence distance matrix.

3.10 DESIGN OF *P. INTERMEDIA* AND *P. NIGRESCENS* SPECIFIC OLIGONUCLEOTIDES

The entire 16S rRNA gene sequences of *P. intermedia* and *P. nigrescens* available in the EMBL database were analysed for suitable regions to exploit as species-specific oligonucleotides for PCR.

3.10.1 USE OF EMBL DATABASE AND SELECTION OF SEQUENCES

The EMBL DNA Database was accessed via Seqnet, Daresbury. A search was performed using keywords for 'Sequence-all text': *Prevotella* and 'Sequence-features': 16S. Forty-seven entries matched these search criteria. Five complete 16S rRNA sequences were found for *P. intermedia* ATCC 25611 (L16468 and X73965), *P. nigrescens* NCTC 9336 (X73963), *P. nigrescens* ATCC 33563 (L16471) and *P. nigrescens* ATCC 25261 (L16479). The accession numbers are shown in parentheses.

These sequences were aligned and compared using the command PILEUP. The command PRETTY with a plurality of 5, produced a consensus sequence for all five chosen entries. A consensus sequence was produced and regions displaying a lack of identity between the 2 species were examined.

Initially, 16 regions with lengths of 19-36 bases were chosen. The first 300 bp of the 16S rRNA gene contained 3 *P. intermedia* sequences (pint-1; 21 bases, pint-2; 24 bases and pint-3; 26 bases) and 3 *P. nigrescens* sequences (pnig-1; 21 bases, pnig-2; 22 bases and pnig-3; 26 bases). The region between 600 bp and 750 bp contained 3 *P. intermedia* (pint-4; 29 bases, pint-5; 24 bases and pint-9; 30 bases) and 3 *P. nigrescens* sequences (pnig-4; 29 bases, pnig-5; 24 bases and pnig-9; 30 bases). The remaining sequences were chosen between 1100 bp and 1300 bp, 2 *P. intermedia* (pint-6; 36 bases and pint-7; 19 bases) and 2 *P. nigrescens* (pnig-7; 23 bases and pnig-8; 23 bases).

3.10.2 COMPARISON OF POTENTIAL PRIMER SEQUENCES TO 16S RRNA GENE SEQUENCES OF OTHER ORAL BACTERIAL SPECIES

The 16S rRNA gene sequences of those oral microorganisms listed in table 6.3 (appendix 6.11) were aligned and a lack of homology to the potential sequences was confirmed.

3.10.2.1 *P. intermedia* oligonucleotide sequences

All *P. intermedia* sequences displayed some degree of identity to the 16S rDNA of other *Prevotella* spp. The number of mismatches seen was within the range of 1-14 base

differences. Sequences pint-1, pint-2 and pint-3 showed the lowest level of identity with other *Prevotella*, whilst pint-7 and pint-9 showed the most. Some sequence conservation was seen with other oral species, but the number of base differences was higher, within the range of 2-20 bases.

3.10.2.2 *P. nigrescens* oligonucleotide sequences

All *P. nigrescens* sequences displayed some degree of identity to the 16S rDNA of other *Prevotella* spp., with mismatches within the range of 2-13 base differences. Sequence pnig-1 showed the lowest level of identity with other *Prevotella* spp., whilst pnig-7 showed the highest. Some sequence conservation was seen with other oral species, although the range of base differences was higher, 4-20 bases.

3.10.3 FORMATION OF STEMLOOP STRUCTURES

The ability to form undesirable stemloop structures was tested through Seqnet, Daresbury, using the STEMLOOP command. Sequences that did not form any stemloop structures were considered desirable.

P. intermedia sequences pint-1, pint-2, pint-6, pint-9 and *P. nigrescens* sequences pnig-1, pnig-2, pnig-5, pnig-8 and pnig-9 formed zero stemloops.

P. intermedia sequences pint-3, pint-5, pint-7 and *P. nigrescens* sequences pnig-3, pnig-4 and pnig-7 formed 1 stemloop.

P. intermedia sequence pint-4 formed 3 stemloops.

3.10.4 FASTA SEARCH

Potential sequences were examined by FASTA search and sequences that displayed high levels of identity to either *P. intermedia* or *P. nigrescens* (according to the sequence under test), other *Prevotella* or oral species were discounted.

3.10.4.1 *P. intermedia* oligonucleotide sequences

Sequences pint-5 and pint-9 showed a high level of identity (a maximum of 6 mismatches throughout its length for pint-5 and 4 at the ends of the sequence for pint-9) with many *Prevotella* species. Pint-5 displayed high levels of identity to *P. disiens*, *P. corporis*, *P. oris*, *P. dentalis* and *P. nigrescens* and pint-9 displayed identity to *P. buccalis*, *P. melaninogenica* and *P. loescheii* as well as *Porphyromonas asaccharolytica* and *B. fragilis*. Sequence pint-6 displayed one terminal mismatch to the 16S rRNA gene from *P. disiens*

and three throughout the length to *P. melaninogenica* and pint-1 was seen to have only 3 mismatches throughout its length to *Peptostreptococcus micros*. Sequence pint-4 showed high levels of identity to *P. pallens*, *P. corporis* and *P. disiens*. Sequence pint-7 displayed 100% identity to 16S rDNA from *P. disiens*. FASTA searches on sequences pint-2 and pint-3 revealed few bacterial matches and none were considered significant, yeast and viral matches predominated.

3.10.4.2 *P. nigrescens* oligonucleotide sequences

Sequences pnig-2 and pnig-3 displayed homology to *P. loescheii* with the mismatches in a terminal position, which would render hybridization with these sequences less stable. In contrast, sequence pnig-9 had 3 base mismatches over 30 bp to *P. loescheii* and *P. gingivalis* sequences. The mismatches were evenly spaced throughout the length which would potentially have allowed hybridization to occur and be stable. Pnig-7 showed 100% identity (zero mismatches) over 23 bp with *P. melaninogenica*. Two mismatches including a deletion (in the *P. nigrescens* sequence, which would not be present in the oligonucleotide when used for PCR) were seen close to the end for *P. denticola* ATCC 35308 and ATCC 33185; this was also true of *P. buccae* ATCC 33574 and ATCC 33690 and *P. dentalis*. Sequence pnig-5 displayed identity to 16S rDNA from *B. fragilis*, pnig-4 had only 1 mismatch with '*B. intermedius*' rDNA and 3 terminal mismatches with *P. pallens* whilst p-nig 8 displayed a high degree of complementarity to *P. melaninogenica* (1 mismatch). Only pnig-1 demonstrated no identity to other *Prevotella* species or oral bacteria, with only low levels to plant viruses and *Escherichia coli*.

3.10.5 POTENTIAL SEQUENCES

The FASTA searches and stemloop formation were considered together to rule out sequences for use as PCR primers. Sequence pint-2 and pnig-1 were chosen due to the low levels of identity to other *Prevotella* species and oral organisms and the absence of stemloop formation and are referred to as P-int and P-nig. These were compared to 1Bi-1 and 2Bi-1 (Dix *et al.*, 1990; Shah *et al.*, 1995) and all 4 sequences were used as one of a pair of PCR primers with RE-TPU1 (Choi *et al.*, 1994). Primer sequences are listed in table 2.4.

3.11 USE OF SPECIES-SPECIFIC OLIGONUCLEOTIDES AS PCR PRIMERS

Bands of approximately 204 bp (P-int); 228 bp (P-nig); 499 bp (1Bi-1) and 488 bp (2Bi-1) were expected when the 4 sequences were used as one of a primer pair. The annealing position of the primers is illustrated in the sequence alignment shown in appendix 6.12.

The optimal annealing temperature and primer and magnesium concentrations were determined for each primer pair as described in sections 2.8.3.1 and 2.8.3.2. At the initial annealing temperature of 57°C, amplification of DNA from both *P. intermedia* and *P. nigrescens* as well as from *P. gingivalis* was seen for all four primers. The annealing temperature was increased in 3° increments, until the optimum appeared to have been passed, then single degree changes were tested. Magnesium concentrations in the range 1.5 mM - 5 mM and primer concentrations in the range 0.1 μM - 0.5 μM were tested. For all primers, amplification was seen with the expected templates between primer concentrations of 0.2 - 0.5μM. An increase in product intensity with increasing primer concentration was seen. The optimal conditions used for subsequent analysis are summarised in table 3.2.

3.11.1 PCR OPTIMISATION

3.11.1.1 *P. intermedia* primer P-int and RE-TPU1

At annealing temperatures of 60°C, 63°C and 66°C amplification of *P. nigrescens* ATCC 25261 and ATCC 33563 and *P. gingivalis* ATCC 33227 was seen in addition to *P. intermedia*. At 69°C, amplification of duplicate *P. intermedia* ATCC 25611 was variable. Dropping the annealing temperature to 67°C resulted in specific amplification.

A magnesium titration was performed at an annealing temperature of 67°C. Above a $MgCl_2$ concentration of 1.5 mM (up to 3.0 mM) some weak amplification products of *P. nigrescens* ATCC 33563 were seen; these disappeared above 3.5 mM.

3.11.1.2 *P. nigrescens* primer P-nig and RE-TPU1

At an annealing temperature of 60°C, the only cross reactivity came from *P. intermedia*. Raising the annealing temperature to 63°C removed this amplification product but DNA from *A. actinomycetemcomitans* ATCC 29525 was amplified. Increasing the temperature further to 66°C removed all amplification products apart from a faint band due to the amplification of *P. nigrescens*. The annealing temperature was then dropped back to 64°C. Although the major bands resulted from the amplification of *P. nigrescens* DNA, faint products were still seen for *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 33227 and *A.*

actinomycetemcomitans ATCC 29525. Increasing the magnesium concentration (see section 3.11.1.2.2) from 1.5 mM to 2.5 mM, decreased the non-specific products further, and raising the annealing temperature to 65°C removed them totally.

A magnesium titration was performed at a non-specific annealing temperature of 63°C. A concentration of 2.5 mM was decided upon, which gave a strong amplification product with *P. nigrescens* template only when an annealing temperature of 65°C was used.

Table 3.1 Optimal PCR conditions determined for species-specific PCR primers and used for all analyses

	P-int	P-nig	1Bi-1	2Bi-1
Annealing temperature (°C)	67	65	68	69
Primer concentration (μM)	0.3	0.3	0.3	0.3
Magnesium concentration (mM)	1.5	2.5	2.0	2.0

3.11.1.3 *P. intermedia* primer 1Bi-1 and RE-TPU1

At an annealing temperature of 60°C, amplification of *P. nigrescens* ATCC 25261 and ATCC 33563 and *P. gingivalis* ATCC 33227 was seen in addition to *P. intermedia*. Raising the temperature to 63°C resulted in amplification of *A. actinomycetemcomitans* ATCC 29525 instead of *P. gingivalis* ATCC 33227. At 66°C, the only cross reactivity came from *P. nigrescens* ATCC 33563 and at 69°C no amplification was seen. A temperature of 67°C was too low with *P. nigrescens* ATCC 33563 still amplified. However, those products were removed at 68°C. A magnesium concentration of 2.0 mM was used, giving a strong amplification product with *P. intermedia* template when a non-specific annealing temperature was used (66°C)

3.11.1.4 *P. nigrescens* primer 2Bi-1 and RE-TPU1

At annealing temperatures of 60°C and 63°C, amplification of *P. nigrescens* ATCC 25261 and ATCC 33563 was seen as well as *P. gingivalis* ATCC 33227 and *A. actinomycetemcomitans* ATCC 29525. Using an annealing temperature of 66°C, amplification products were seen for all templates other than *C. ochracea* W42. These products were lost at 69°C when 2.0mM $MgCl_2$ was used.

Amplification of both *P. nigrescens* type strains was seen at 2.0 mM, 3.5 mM and 4.5 mM $MgCl_2$. There was no amplification at 1.5 mM, 2.5 mM, 4.0 mM and 5.0 mM and a value of 2.0 mM was chosen.

3.11.2 AMOUNT OF DNA TEMPLATE

All four primers were tested as described in section 2.8.5, against approximately 20 ng, 60 ng, 80 ng and 100 ng template DNA from *P. intermedia* ATCC 25261, *P. nigrescens* ATCC 33563, *P. nigrescens* ATCC 25611, *P. gingivalis* ATCC 33227, *C. ochracea* W42 and *A. actinomycetemcomitans* ATCC 29525.

3.11.2.1 *P. intermedia* primers P-int and 1Bi-1

Primers P-int and 1Bi-1 exhibited species-specific amplification (*P. intermedia*) from 20-80 ng of template DNA; no amplification was seen with 100 ng of template DNA. No cross reactivity was seen with the other periodontal pathogens. Figure 3.15 illustrates this with primer P-int.

3.11.2.2 *P. nigrescens* primers P-nig and 2Bi-1

Primers P-nig and 2Bi-1 exhibited inconsistent amplification. Primer P-nig amplified 20-60 ng of template from *P. nigrescens* ATCC 33563 and 40-100 ng of template from *P. nigrescens* ATCC 25261. Non-specific amplification was also seen; primer P-nig amplified 40 ng and 80 ng of template from *P. intermedia* and 2Bi-1 amplified 80 ng. No cross reactivity was seen with the other periodontal pathogens.

3.11.3 SPECIFICITY OF PCR REACTION

Approximately 40 ng of DNA from known strains of *P. intermedia* and *P. nigrescens*, Gram-negative black-pigmenting clinical isolates, *P. gingivalis* ATCC 33227, *C. ochracea* W42, *A. actinomycetemcomitans* ATCC 29525, *P. pallens*, *P. corporis* and PINLO strains was used to test the specificity of P-int and P-nig.

3.11.3.1 *P. intermedia* primer P-int and RE-TPU1

Only *P. intermedia* DNA from strains *P. intermedia* ATCC 25611 and MH3 was amplified by this primer. No amplification of *P. intermedia* strains MH6, MH12 and MH15 was seen. Two clinical isolates (B and C) were amplified during both experiments by this primer and identified as *P. intermedia* (see results section 3.15.5). There was no amplification of *P. nigrescens*, *P. pallens*, *P. corporis* or PINLO strains in either of the occasions the primer was tested.

3.11.3.2 *P. nigrescens* primer P-nig and RE-TPU1

P. nigrescens strains MH1, MH2 and MH5 were amplified consistently whilst *P. nigrescens* LM94, ATCC 25261 and ATCC 33563 were amplified inconsistently. The 2 clinical isolates (A and D) were also amplified by this primer and identified as *P. nigrescens* (see results section 3.15.5). Amplification of *P. intermedia* MH3 and MH15, *P. pallens* AHN 9423, *P. corporis* ATCC 33547, and *P. corporis* A350 was also seen during the 2 experiments. One PINLO strain, HST 1156 was also amplified by this primer during both experiments.

3.11.4 MULTIPLEX PCR

Multiplex PCR was performed due to inconsistent and non-specific amplification (see section 3.11.3) of the PCR primers. The priming and subsequent amplification of the universal bacterial primers RE-TPU1 and RE-RTU3 was tested in combination with the test primer and RE-TPU1 using both 40 and 80 ng of template DNA.

3.11.4.1 *P. intermedia* primer P-int

Primer P-int was tested and the dominant product was an approximate 204 bp band due to amplification with P-int and RE-TPU1, although a faint band at approximately 500 bp due to amplification with RE-TPU1 and RE-RTU3 was seen.

3.11.4.2 *P. nigrescens* primers P-nig and 2Bi-1

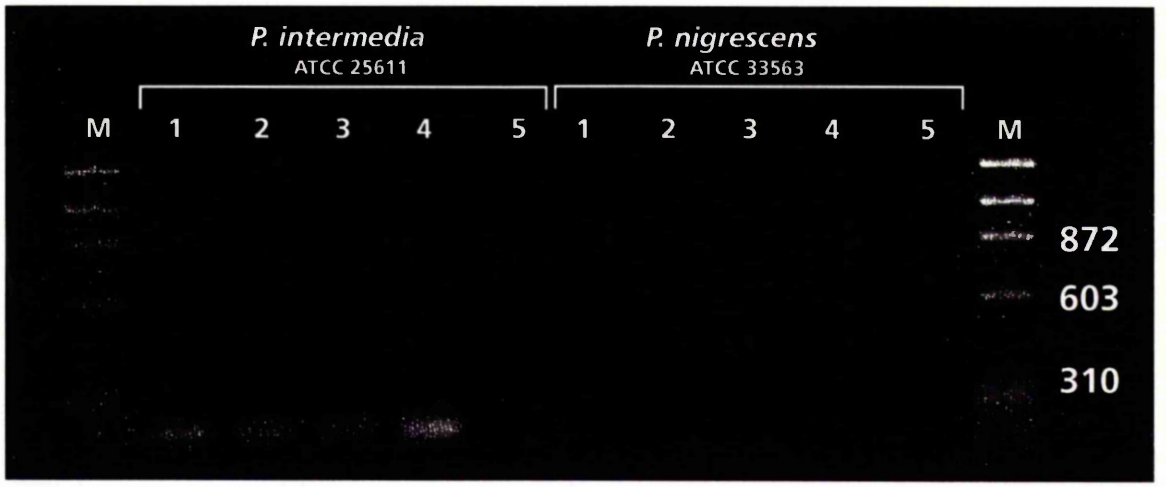
When multiplex PCR was performed using equal amounts of all three primers including P-nig, the dominant product was an approximate 500 bp band due to amplification by RE-TPU1 and RE-RTU3. This was seen for all templates (*P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *P. nigrescens* ATCC 25261 and *P. gingivalis*). A minor product of the other primer pair was seen. When half as much RE-RTU3 was used, the dominant product was still a 500 bp one. However, the product (approximately 228 bp) due to amplification with P-nig and RE-TPU1 was much stronger and was only seen in the four lanes belonging to *P. nigrescens* ATCC 33563 at 40 and 80 ng and *P. nigrescens* ATCC 25261 at 40 ng and 80 ng. A third larger product of weak intensity is also seen in these four lanes. This is illustrated in figure 3.16.

The same experiments were repeated with primer 2Bi-1 and inconsistent amplification of *P. nigrescens* species DNA was seen regardless of the amount of RE-RTU3 used. When amplification did occur, the only product was the larger 500 bp band resulting from amplification by the universal sequencing primers.

3.11.5 VARIATIONS IN AMPLIFICATION AS A RESULT OF INCONSISTENCIES IN TEMPERATURE PROFILE ACROSS PCR THERMAL CYCLER

The consistency of amplification was tested with primers P-int (template 40 ng of *P. intermedia* ATCC 25611) and P-nig (template 40 ng of *P. nigrescens* ATCC 25261) independently by placing 8 PCR reactions for each primer in specified sites within the Crocodile II™ thermal cycler (Appligene). In both cases two reactions placed in the same site showed no amplification at all. These sites were farthest away from the temperature probe. However, in the case of P-nig, an additional site was not amplified, which was closer to the temperature probe than the other two (data not shown).

Figure 3.15 PCR with primer P-int using template DNA concentrations of 20-100 ng

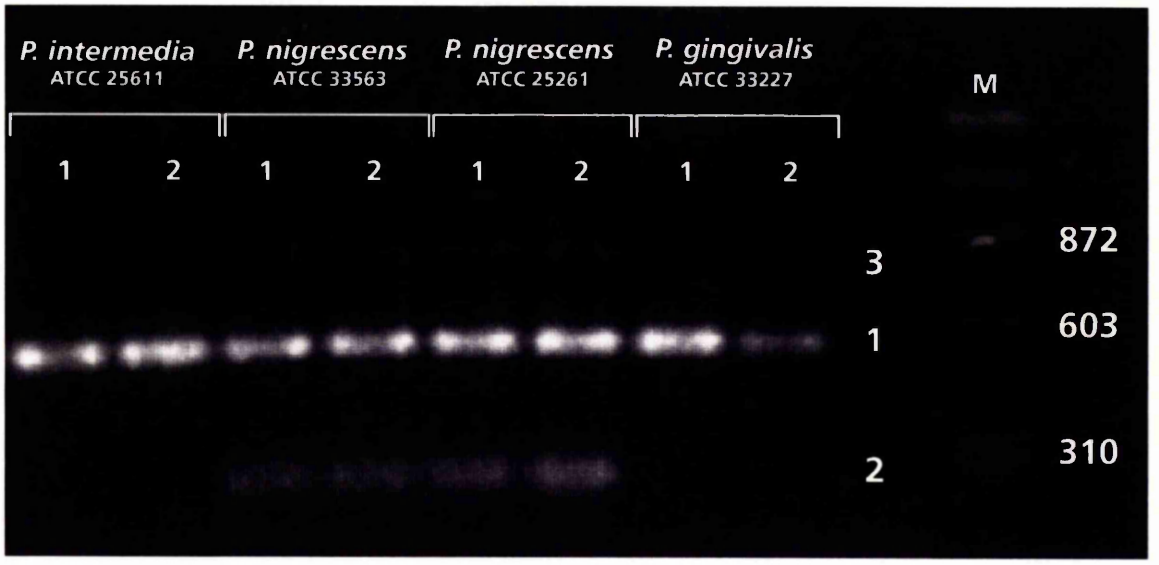


KEY:

Numbers 1-5, approximate DNA concentrations. 1, 20 ng; 2, 40 ng; 3, 60 ng; 4, 80 ng; 5, 100 ng

M, molecular weight marker (ϕX174 DNA *Hae* III digest)

Figure 3.16 Multiplex PCR using primer pairs P-nig and RE-TPU1 and RE-TPU1 and RE-RTU3



KEY:

Numbers 1 and 2, approximate DNA concentrations. 1, 40 ng; 2, 80 ng;

A, band due to amplification with primers RE-TPU1 and RE-RTU3; B, band due to amplification with primers P-nig and RE-TPU1

M, molecular weight marker (ϕX174 DNA *Hae* III digest)

RESULTS

SECTION B: CLINICAL MICROBIOLOGY (PLS)

Please note that this section contains a detailed explanation of results at all stages of the identification procedure, as well as some of the rationale behind the next step taken in the identification process to. This is intended to provide the reader with a clear picture of why certain steps were taken and final identifications accepted. All clinical isolates are discussed in terms of reference number only, final identifications are related to the patient in section 4.14 of the discussion.

3.12 IDENTIFICATION OF AEROBIC (AND FACULTATIVE) MICROORGANISMS FROM PATIENTS WITH PLS

3.12.1 COMMERCIAL IDENTIFICATION KITS

Of the 53 isolates labelled aerobic, 52 were tested with commercial identification kits (API 20 NE; API NH, Rapid ID 32 Strep; API Coryne; Rapid ID 32 A; RapID ANA II) this included 2 isolates labelled as **4** *1C* (I and II). Table 3.3 lists the results obtained which do not necessarily represent final identifications, which take into account other factors, covered in subsequent sections. Thirteen out of 52 cultures labelled as aerobic and facultative were tested with more than one identification kit, when either an unacceptable identification or no identification was obtained. However, 22 out of the 52 isolates in table 3.3 were tentatively identified by commercial identification kits alone, where a positive identification with >79.9% probability of correct identification was obtained. Four isolates (**4** *2A*, **5** *1C*, **6** *1A* and 7 *1B*) were identified using API NH to genus level as *Neisseria* species (with identity levels of 97.5 - 99.8%) and four isolates were identified using RapID ANA II system, three (**2** *1A*, **8** *1A* and **10** *1A*) as *Capnocytophaga* species (with identity levels of 99.9%) and one as *Mobiluncus* sp. (**2** *1B*). Fourteen isolates (**1** *1C*, **2** *1C*, **2** *1D*, **2** *2A*, **3** *1A*, **3** *1D*, **3** *1F*, **4** *1B*, **4** *1F*, **6** *2B*, **8** *1B*, **9** *1B*, **11** *2A* and **12** *1A*) were identified by Rapid ID 32 Strep. In all cases, except one, identification was made to species level, including *S. constellatus*, *S. sanguis*, *S. mitis* and *Gemella haemolysans*. One isolate, **6** *2B* was identified as *Lactococcus* species (*Lc. lactis cremoris*), one was identified only as a *Streptococcus* species and **9** *1B* reached an identification level of 79.9%, which was accepted. These isolates are marked in table 3.3 by symbols which cross-reference with tables (6.12, 6.13 and 6.14) found in appendix 6.17.1, depicting individual test results.

Of the remaining 31 isolates, 11 (**1** *1A*, **3** *1B*, **4** *1A*, **4** *1C* II, **4** *2G*, **5** *1A*, **6** *2A*, 7 *2F*, **8** *2A*, **9** *2A*, **14** *2A*) gave good (above 79.9%) level identifications with commercial identification kits. However, all were later re-examined on the basis of further tests described in section

3.7.2 onwards. A further 12 isolates (**1** *1D*, **1** *1E*, **2** *1F*, **3** *1C*, **3** *2A*, **5** *2A*, **6** *1B*, **9** *2B*, **10** *2A*, **13** *1A*, **14** *1A* and **16** *1A*) gave low level identifications (below 79.9%) which were not accepted initially. In 8 out of these 12 cases, subsequent tests were performed. Seven isolates (**1** *1F*, **1** *2B*, **3** *1E*, **4** *1C* I, 9 *2E*, **9** *2F* and **11** *1B*) tested gave no result (labelled unacceptable profile or questionable code in table 3.3) and 1 isolate **3** *2B* was not tested due to loss of viability.

3.12.2 SUBSEQUENT IDENTIFICATION

3.12.2.1 Gram stain and cellular morphology

Table 3.4 lists the final Gram stain results for all 53 aerobic and facultative isolates. The results are those obtained during this study, which in all cases (except 2; **5** *2A* and **10** *2A*) correlated with those received from MRI. A total of 35 were Gram-positive, of which 25 were cocci and 10 bacilli. Of the 16 Gram-negative isolates 10 were bacilli and 6 cocci. The Gram stain morphology of 2 isolates is unknown and could not be confirmed due to the loss of viability of these isolates. A complete list of isolates which ceased to be viable before identification could be completed is found in table 6.18, appendix 6.18.

These results were used in combination with those obtained by commercial identification kits to accept or disprove those identifications over 79.9%.

Table 3.3 List of aerobic (and facultative) clinical isolates and the commercial identification kits used for identification

A key to symbols is provided at the end of this table.

CLINICAL ISOLATE	BIOCHEMICAL KIT(S) USED	RESULTS	% ID	LEVEL
1 *1A*	API CORYNE	Unacceptable profile	—	—
	RAPID ANA II †	*Clostridium difficile*	99.95	Implicit
1 *1C*	RAPID ID 32 STREP	*Streptococcus bovis II* *S. saliv. salivarius*	84.8 12.8	Good ID to genus ⊕
1 *1D*	RAPID ID 32 STREP	*S. pneumoniae* *Gemella morbillorum* *Streptococcus* sp.	67.4 14.4 16.7	Identification not valid
1 *1E*	RAPID ID 32 A ‡	*Actinomyces meyeri* *Capnocytophaga* sp.	76.5 23.4	Low discrimination
1 *1F*	RAPID ANA II †	Unacceptable profile	—	—
1 *2B*	RAPID ANA II †	Questionable code	—	—
2 *1A*	RAPID ANA II †	*Capnocytophaga* sp.	99.99	Satisfactory ⊗
2 *1B*	API CORYNE RAPID ANA II †	Unacceptable profile *Mobiluncus* sp.	— 99.9	— Satisfactory ⊗
2 *1C*	RAPID ID 32 STREP	*S. constellatus*	99.5	Very good ID ⊕
2 *1D*	RAPID ID 32 STREP	*Gemella morbillorum*	95.6	Doubtful profile ⊕
2 *1F*	API 20 NE	*Listonella damsela* *Pasteurella* sp. *Aeromonas* sp.	64.4 27.8 6.6	Doubtful profile
2 *2A*	RAPID ID 32 STREP	*S. saliv. salivarius*	97.9	Good ID ⊕
3 *1A*	RAPID ID 32 STREP	*S. mitis 1* *S. oralis* *S. mitis 2*	82.7 14.7 2.2	Very good ID to genus ⊕
3 *1B*	RAPID ANA II † API CORYNE	*Clostridium hastiforme* Unacceptable profile	99.57 —	Implicit —
3 *1C*	RAPID ID 32 STREP	*S. mitis* *S. oralis* *S. pneumoniae*	47.0 26.2 23.8	Good ID to genus
3 *1D*	RAPID ID 32 STREP	*Gemella haemolysans*	99.6	Very good ID ⊕
3 *1E*	API 20 NE RAPID ID 32 A ‡	Unacceptable profile Unacceptable profile	— —	— —
3 *1F*	RAPID ID 32 STREP	*S. mitis* *S. sanguis*	90.0 8.4	Good ID to genus ⊕

CLINICAL ISOLATE	BIOCHEMICAL KIT(S) USED	RESULTS	% ID	LEVEL
3 *2A*	RAPID ID 32 A ‡	*Eubacterium lentum*	38.6	Doubtful profile
		Bac. ureolyticus	19.8	
		Cl. difficile	15.8	
		Pstr. anaerobius	10.9	
	RAPID ANA II †	Unacceptable code	—	—
4 *1A*	API NH	*Neisseria cinerea*	95.9	Excellent ID to genus
	RAPID ID 32 A ‡	*Pstr. micros*	67.2	Doubtful profile
		Actinomyces meyeri	20.0	
		A. odontolyticus	8.8	
		Gemella morbillorum	3.7	
4 *1B*	RAPID ID 32 STREP	*S. constellatus*	99.5	Very good ID ⊕
4 *1C*	RAPID ID 32 STREP	Unacceptable profile	—	—
	RAPID ID 32 STREP	*Leuconostoc* sp.	99.7	Doubtful profile
4 *1F*	RAPID ID 32 STREP	*S. saliv. salivarius*	81.9	Low discrimination ⊕
		Gemella morbillorum	17.1	
4 *2A*	API NH	*N. cinerea*	95.9	Excellent ID to genus ⊖
4 *2G*	RAPID ANA II †	*A. meyeri*	92.42	Probability overlap
		Prop. granulosum	7.55	
	API NH	Unacceptable profile	—	—
5 *1A*	RAPID ID 32 A ‡	*Actinomyces israelii*	69.0	Doubtful profile
		Cl. bifermentans	23.8	
		Gemella mobillorum	6.0	
	RAPID ANA II †	*Cl. linosum*	99.82	Presumptive
5 *1C*	API NH	*Neisseria* sp.	99.8	Very good ID ⊖
5 *2A*	API NH	*Haemophilus aphrophilus / paraphro.*	57.5	Excellent ID to genus
		H. parainfluenzae	42.4	
6 *1A*	API NH	*Neisseria* sp.	97.5	Good ID ⊖
6 *1B*	RAPID ID 32 STREP	*S. constellatus*	38.1	Doubtful profile
		S. anginosus	38.0	
		(S. milleri group)	(76.1)	
		S. agalactiae	23.6	
6 *2A*	RAPID ID 32 STREP	*Leuconostoc* sp.	99.6	Very good ID
6 *2B*	RAPID ID 32 STREP	*Lc. lactis cremoris*	99.9	Excellent ID ⊕
7 *1B*	API NH	*Neisseria* sp.	99.8	Very good ID ⊖
7 *2F*	RAPID ANA II †	*Ps. micros*	91.9	Inadequate. Genus level ID
		Ps. magnus	0.78	
8 *1A*	API NH	Unacceptable profile	—	—
	RAPID ANA II †	*Capnocytophaga* sp.	99.99	Satisfactory ⊗
8 *1B*	RAPID ID 32 STREP	*S. sanguis*	93.6	Good ID ⊕
8 *2A*	RAPID ID 32 A ‡	*Actinomyces meyeri*	99.9	Very good ID

CLINICAL ISOLATE	BIOCHEMICAL KIT(S) USED	RESULTS	% ID	LEVEL
9 *1B*	RAPID ID 32 STREP	*S. oralis* *S. mitis 1* *S. mitis 2*	79.9 19.8 0.1	Very good ID to genus[⊕]
9 *2A*	API NH	*N. cinerea*	95.9	Excellent ID to genus
9 *2B*	RAPID ID 32 STREP	*S. mitis* *S. acidominimus* *S. sanguis*	47.8 27.3 12.6	Doubtful profile
9 *2E*	RAPID ANA II [†]	Questionable code	—	—
9 *2F*	RAPID ANA II [†]	Unacceptable profile	—	—
10 *1A*	API NH RAPID ANA II [†]	Unacceptable profile *Capnocytophaga* sp.	— 99.99	— Adequate [⊗]
10 *2A*	API NH	*Neisseria meningitidis* *N. polysaccharea* *Neisseria* sp.	55.9 29.2 13.3	Doubtful profile
11 *1B*	API CORYNE RAPID ANA II [†]	Unacceptable profile Questionable code	— —	— —
11 *2A*	API NH RAPID ID 32 STREP	*H. aphrophilus / H. paraphrophilus* *H. parainfluenzae* *S. constellatus* *S. anginosus*	57.5 42.4 81.7 18.2	Excellent ID to genus Excellent ID to genus [⊕]
12 *1A*	RAPID ID 32 STREP	*S. constellatus* *Leuconostoc* sp.	82.2 16.1	Good ID [⊕]
13 *1A*	RAPID ID 32 STREP	*S. mitis 1* *S. adjacens* *S. mitis 2*	46.3 37.8 11.3	Good ID to genus
14 *1A*	RAPID ID 32 STREP	*S. mitis 1* *S. mitis 2* *S. oralis* *S. pneumoniae*	40.7 37.4 10.3 7.9	Good ID to genus
14 *2A*	API NH	*N. cinerea*	95.9	Excellent ID to genus [⊕]
16 *1A*	API NH	*Neisseria meningitidis* *N. polysaccharea* *Neisseria* sp.	55.9 29.2 13.3	Doubtful profile

KEY:

A., Actinomyces; Bac., Bacteroides; Cl., Clostridium; H. Haemophilus; Lc., Lactococcus; N., Neisseria; S., Streptococcus; Prop., Propionibacterium; Pstr., Peptostreptococcus

⊕ See table 6.12 (appendix 6.17.1) for individual test results from Rapid ID 32 Strep.

⊖ See table 6.13 (appendix 6.17.1) for individual test results from API NH.

⊗ See table 6.14 (appendix 6.17.1) for individual test results from RapID ANA II system.

† Gram-positive bacilli were tested using the RapID ANA II system to ensure that microaerophilic rods such as *Actinomyces* or *Lactobacillus* were not missed during identification. For a full discussion on this subject see sections 4.9.4 and 4.10.1.3.

‡ Gram-positive bacilli were tested using Rapid ID 32 A to ensure that genera such as *Actinomyces* or *Lactobacillus* were not missed during identification. For a full discussion on this subject see sections 4.9.4 and 4.10.1.3.

FOOTNOTES TO TABLE 3.3.

1. Gram morphologies are listed in table 3.4.
2. A description of rationale behind choice of kit is provided in material and methods section 2.10.1 and discussed in section 4.10.1.3 of the discussion. The reader is referred specifically to section 4.9.4 for an explanation regarding the use of the anaerobe identification kits RapID ID ANA II system and Rapid ID 32 A for these aerobic (and facultative) isolates.
3. The identification achieved is given but may not represent final identifications, which take into account Gram stain morphology (table 3.4), other tests (table 3.5) and partial 16S rRNA gene sequencing (table 3.6). Final identifications are represented in tables 3.7 -3.9.

Table 3.4 Gram stain morphologies for aerobic (and facultative) clinical isolates

These results represent those achieved during this study, the majority of which correspond to those received from staff at MRI.

CLINICAL ISOLATE REFERENCE	GRAM STAIN RESULT
1 *1A*	GPB
1 *1C*	GPC
1 *1D*	GPC
1 *1E*	GNB
1 *1F*	GPB
1 *2B*	GPB
2 *1A*	GNB
2 *1B*	GPB
2 *1C*	GPC
2 *1D*	GPC
2 *1F*	GNB
2 *2A*	GPC
3 *1A*	GPC
3 *1B*	GPB
3 *1C*	GPC
3 *1D*	GPC
3 *1E*	GNB
3 *1F*	GPC
3 *2A*	GNB
3 *2B*	Unknown
4 *1A*	GNB
4 *1B*	GPC
4 *1C* I/II	GPC
4 *1F*	GPC
4 *2A*	GNC
4 *2G*	GPB

CLINICAL ISOLATE REFERENCE	GRAM STAIN RESULT
5 *1A*	GPB
5 *1C*	GNC
5 *2A*	GPC [§]
6 *1A*	GNC
6 *1B*	GPC
6 *2A*	GPB
6 *2B*	GPC
7 *1B*	GNC
7 *2F*	GPB
8 *1A*	GNB
8 *1B*	GPC
8 *2A*	GNB
9 *1B*	GPC
9 *2A*	GNC
9 *2B*	GPC
9 *2E*	GPB
9 *2F*	Unknown
10 *1A*	GNB
10 *2A*	GPC [§]
11 *1B*	GPB
11 *2A*	GPC
12 *1A*	GPC
13 *1A*	GPC
14 *1A*	GPC
14 *2A*	GNC
16 *1A*	GNB

KEY:

GPB, Gram-positive bacilli; GNB, Gram-negative bacilli; GPC, Gram-positive cocci; GNC, Gram-negative cocci; [§] Gram result differs from MRI (**5** *2A* - GNC; **10** *2A* - GNC)

3.12.2.2 Catalase test, oxidase activity, oxidation or fermentation of glucose (Hugh and Leifson), growth in an anaerobic environment and colony colour

The results of catalase and oxidase tests, oxidation-fermentation characteristics, ability to grow in an anaerobic environment and colony colour, are recorded in table 3.5. These characteristics were recorded in 33 cases, with the exception of colony colour which was not recorded in 6 cases. Those excluded were no longer viable and included some for which an identification had been accepted (isolates **2** *1A*, **2** *1C*, **4** *1B*, **6** *2A*, **8** *1A*, **8** *1B* and **10** *1A*).

These results were used in combination with the Gram stain results (table 3.4) and the identification tables given in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) to provide a genus level identification and to support (or not) the results obtained by commercial identification kits.

3.12.2.3 Extracellular polysaccharide production, colony colour, resistance to optochin antibacterial, nitrate reduction and requirement for V-factor

These characteristics were tested in specific cases were they were suggested by API (bioMérieux) after analysis of identification kit profiles. Eight isolates (**4** *1A*, **5** *1C*, **6** *1A*, **7** *1B*, **10** *2A*, **14** *1A*, **14** *2A* and **16** *1A*) giving a possible result with the API NH kit were tested for the ability to reduced nitrates, all were negative. This characteristic was used in combination with colony colour as suggested by bioMérieux as a complementary test to help speciate *Neisseria*. Their guidelines suggest that nitrate reduction is a characteristic of 95% of *N. mucosa* isolates but only 5% of *N. sicca* or *N. subflava* and that 95% of *N. subflava* exhibit yellow colonies compared to 25% of N. mucosa and 5% of *N. sicca*.

The production of extracellular polysaccharides (EPS) dextran/levan by streptococcal species was also suggested as a complementary test by bioMérieux and its production on TYC agar (see appendix 6.1.3.3) was tested for 7 isolates (**3** *1A*, **3** *1C*, **3** *1F*, **4** *1F*, **9** *1B*, **9** *2B* and **14** *1A*). Two isolates (**3** *1C* and **9** *1B*) were positive for EPS production and 5 were negative.

Sensitivity to the antibacterial Optochin is a characteristic of *Streptococcus pneumoniae*; all other streptococcal species are resistant. Sensitivity of one isolate (**1** *1D*) was tested because *S. pneumoniae* was at the top of the identification list using Rapid ID 32 Strep.

Table 3.5 Results of catalase and oxidase tests, oxidation-fermentation characteristics, ability to grow in an aerobic environment and colony colour for each aerobic (and facultative) isolate

CLINICAL ISOLATE REFERENCE	CATALASE TEST	OXIDASE TEST	OXIDATION-FERMENTATION TEST	GROWTH IN ANAEROBIC ENVIRONMENT	COLONY COLOUR
1 *1A*	-	-	NA	+	W
1 *1C*	-	-	F	+	Y
1 *1D*	-	-	F	+	Y
1 *2B*	-	-	F	+	Y-Or
2 *1B*	-	-	NA	+	NT
2 *1D*	-	-	NA	+	NT
2 *2A*	-	-	NA	+	NT
3 *1A*	-	-	NA	+	NT
3 *1C*	-	-	F	+	C
3 *1D*	-	-	NA	+	S-G
3 *1E*	-	+	F	+	NT
3 *1F*	-	-	NA	+	Y
3 *2A*	-	+	NA	-	W
4 *1C* I/II	-	-	NA	+	C-G
4 *1F*	-	-	F	+	C-W
4 *2G*	+	-	NA	-	Or
5 *1C*	+	+	F	-	Y-Br
5 *2A*	-	-	O	-	C
6 *1A*	-	+	NA	+	C-Y
6 *2B*	-	-	NA	+	C
7 *1B*	-	-	NA	+	W
7 *2F*	-	-	F	+	C
9 *1B*	-	-	F	+	C
9 *2A*	+	+	NA	+	Or-Br
9 *2B*	+	-	F	+	C
9 *2E*	-	-	NA	+	W
10 *2A*	-	-	NA	+	C-Br
11 *2A*	-	-	F	+	NT
12 *1A*	-	-	F	+	Y-Br
14 *1A*	-	-	F	+	C-Y
14 *2A*	-	+	NA	+	Or-Br
16 *1A*	-	+	NA	+	W-C

KEY:

C, cream; Br, brown; F, fermentation; G, grey; NA, no action; NT, not tested; O, oxidation; Or, orange; S, silver; W, white; Y, yellow

The percentage likelihood of correct identification was low (67.4%), therefore optochin resistance was used to rule out this identification as no zone of inhibition was seen. In other words, isolate **1** *1D* was optochin resistant and therefore unlikely to be *S. pneumoniae*.

A requirement for V-factor (nicotinamide adenine dinucleotide; NAD^+) is seen by strains of *Haemophilus*; it was tested for when isolates identified using API NH had *Haemophilus* spp. in the list of taxa, although in all cases the percentage identity was below 79.9%. Three isolates were tested (**5** *2A*, **6** *2B* and **11** *2A*) as described in materials and methods section 2.10.2.7. None of the isolates displayed satellitism of colonies around *S. aureus* or increased colony size, suggesting these isolates were not *Haemophilus* spp.

These results were used in combination with the Gram stain results (table 3.4), characteristics tested in section 3.12.2.-3.1.2.4 (table 3.5) and the identification tables given in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) to provide a genus level identification and to asses the sensibility of results obtained by commercial identification kits.

3.12.2.4 Spore stains

One isolate identified as *Clostridium* spp. (**1** *1A*; 99.95%) and one isolate where *Clostridium* appeared within the list of suggested taxa (**3** *2A*; 15.8%) were stained for the production of spores using malachite green. No spores were seen when 5 day old cultures were tested. Isolates **3** *1B* and **5** *1A* were also identified as *Clostridium* spp. but loss of viability prevented these being stained for spore production.

3.12.3 PARTIAL SEQUENCING OF 16S RRNA GENE

3.12.3.1 Isolates which were sequenced

Eighteen isolates (**1** *1A*, **1** *1D*, **1** *2B*, **2** *1B*, **3** *1C*, **3** *1E*, **3** *2A*, **4** *1C* I, **4** *1C* II, **5** *2A*, **6** *2B*, **7** *2F*, **9** *2A*, **9** *2E*, **10** *2A*, **11** *2A*, **14** *2A* and **16** *1A*) were subjected to DNA extraction and partial sequencing of the 16S rRNA gene. Five (**1** *1A*, **4** *1C* II, **7** *2F*, **9** *2A* and **14** *2A*) out of the 18 isolates had been identified (above 79.9%) by identification kits and had the identification discounted by Gram morphology or other biochemical tests and only 1 isolate (**4** *1C* II) could be assigned to a genus. Six isolates (**1** *1D*, **3** *1C*, **3** *2A*, **5** *2A*, **10** *2A* and **16** *1A*) gave unacceptable identifications using commercial kits but 5 (except **3** *2A*) were identifiable to genus level based on Gram morphology or other tests. Isolates **1** 1*F*, **1** *2B*, **3** *1E*, **4** *1C* I, **9** *2E*, **9** *2F* and **11** *1B* were unidentified by commercial identification kits, of

these, 3 isolates were non-viable (**1** *1F*, **9** *2F* and **11** *1B*). Four isolates (**1** *2B*, **3** *1E*, **4** *1C* I and **9** *2E*) remained viable for DNA extraction and partial 16S rRNA gene sequence analysis, of which only one was identifiable to genus level (isolate **4** *1C* I) on the basis of phenotypic characteristics and biochemical tests. In addition to isolates remaining unidentified, three isolates (**2** *1B*, **6** *2B* and **11** *2A*) identified using RapID ANA II and Rapid ID 32 Strep as far as the genera *Mobiluncus*, *Lactococcus* and *Streptococcus* respectively, were examined by sequencing.

3.12.3.2 Suggested identifications of the isolates to be sequenced

Both isolates **1** *1A*, which had been discounted as a clostridium and **3** *2A* a Gram-negative rod were tested for catalase, oxidase, growth in an anaerobic environment and oxidation-fermentation characteristics. The results obtained from these tests failed to permit assignment of these isolates to a genus using the first stage table for Gram-positive bacteria from Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993). Isolate **7** *2F* matched 5 genera by this method, none of which correlated with the result obtained by commercial identification kit. The Gram-negative bacillus **16** *1A* exhibited characteristics of the genera *Eikenella* and *Kingella*. As a doubtful profile had been achieved by API NH, a 500 base pair region of the 16S rRNA gene was sequenced.

Gram-negative coccoid isolates **9** *2A* and **14** *2A* were identified as *N. cinerea*. This identification was questioned due to the positive growth of both isolates under anaerobic conditions and the negative catalase test of **14** *2A*.

The two isolates of **4** *1C* were treated separately; both had negative catalase and oxidase activity and grew under anaerobic conditions, characteristics of *Streptococcus*, *Gemella* and *Leuconostoc* spp. Only **4** *1C* II was identified by a kit and both **4** *1C* I and **4** *1C* II were sequenced. Isolates **1** *1D*, **3** *1C*, **5** *2A* and **10** *2A* also exhibited the characteristics and Gram morphology of the genera *Streptococcus*, *Leuconostoc* and *Gemella* but no identification was made by the Rapid ID 32 Strep kit. Isolate **10** *2A* had been tested incorrectly by API NH due to poor a Gram stain, the Gram morphology as well as negative catalase and oxidase tests and the ability to grow in an anaerobic environment ruled out *Neisseria* species. **5** *2A* was also tested incorrectly using API NH, the Gram morphology and lack of requirement for V-factor discounted this identification. **3** *1C* was named *S. mitis* and the production of EPS strengthened this identification, although the percentage identity value

was too low to accept, this isolate was nevertheless classified as a streptococcus. Therefore, these 4 isolates were examined by partial 16S rRNA gene sequencing.

3.12.3.3 Analysis of sequence data

Partial 16S rRNA gene sequences were obtained for 18 aerobic and facultative isolates. A FASTA search was used to provide the most likely phylogenetic relationship and this was checked against the results of the commercial identification kits (table 3.3) Gram stains (table 3.4) and other tests (table 3.5) described in sections 3.12.1 and 3.12.2. Table 3.6 compares the results obtained by commercial identification kits and partial 16S rRNA gene sequencing. In 5 cases (**1** *1D*, **3** *1C*, **9** *2A*, **11** *2A* and **14** *2A*), the genus level identification achieved by commercial identification kits and physiological tests matched the genus suggested by partial 16S rRNA gene sequencing. The Gram morphology of four isolates (**1** *2B*, **3** *2A*, **9** *2E* and **14** *2A*) did not agree with the identification obtained by 16S rRNA gene sequencing. The sequence information of 4 isolates (**1** *1A*, **2** *1B*, **4** *1C* **I** and 7 *2F*) was discarded due to the improbable identifications of *Pseudomonas azotoformans* and *P. olveovorans*, thought to be the result of contamination. As a result identifications were not obtained for 3 isolates, whilst the original identification of *Mobiluncus* achieved using the RapID ANA II system was accepted for isolate **2** *1B*.

Partial 16S rRNA gene sequence information is given in appendix 6.21.1.

Table 3.6 Comparison of identifications of aerobic and facultative isolates obtained by commercial identification kits and partial 16S rDNA sequencing

CLINICAL ISOLATE REFERENCE	COMMERCIAL KIT IDENTIFCATION	PARTIAL 16S RRNA SEQUENCING IDENTIFICATION
1 *1A*	*Clostridium difficile*	*P. azotoformans*
1 *1D*	*Streptococcus* sp.	*S. sanguis*
1 *2B*	No Identification	*S. gordonii*
3 *1C*	*Streptococcus* sp.	*S. sanguis*
3 *1E*	No Identification	*K. indologenes*
3 *2A*	No Identification	*N. elongata*
4 *1C* I	No Identification	*P. azotoformans*
4 *1C* II	*Leuconostoc* sp.	*Stom. mucilaginosus*
5 *2A*	*Haemophilus* sp.	*S. anginosus*
6 *2B*	*Lactococcus lactis cremoris*	*S. milleri* group
7 *2F*	*Peptostreptococcus micros*	*P. olveovorans*
9 *2A*	*N. cinerea*	*N. flavescens*
9 *2E*	No Identification	*L. mesenteroides*
10 *2A*	*Neisseria* sp.	*S. sanguis*
11 *2A*	*S. constellatus*	*S. anginosus*
14 *2A*	*N. cinerea*	*N. flavescens*
16 *1A*	*Neisseria* sp.	*K. dentrificans*

KEY:

N., Neisseria; K., Kingella; L., Leuconostoc; P., Pseudomonas; S., Streptococcus; Stom, Stomatococcus

FOOTNOTES TO TABLE 3.6.

1. Those isolates listed as No Identification, produced either 'questionable code' with RapID ANA II system or 'unacceptable profile' with API 20 NE or Rapid ID 32 A (see table 3.3). Gram-positive bacilli were tested using the RapID ANA II system or Rapid ID 32 A to ensure that microaerophilic rods such as *Actinomyces* or *Lactobacillus* were not missed during identification. The reader is referred specifically to section 4.9.4 for an explanation regarding the use of the anaerobe identification kits for aerobic (and facultative) isolates.
2. With the exception of isolates **5** *2A* and **16** *1A*, all commercial identifications listed were above 79% probability of correct identification.
3. The reason for the choice of commercial identification kit is discussed in sections 2.10.1 and 4.10.1.3.
4. The reader is referred to tables 3.3 for identification kit results, 3.4 for the Gram morphologies of these isolates and 3.5 for results of other tests.
5. An explanation for the reasons that these isolates were subjected to sequence analysis rather than having the identifications accepted is provided in section 3.12.3.2.
6. The suggested identifications from sequence analysis were compared with all previous test results and they are discussed in relation to these and with reference to the final identification in section 3.12.4.
7. *P. azotoformans* and *P. olveovorans* were the result of contamination during the sequencing process, for further details see section 4.11.11.
8. A discussion of 16S rRNA gene sequencing in relation to bacterial identification can be found in section 4.5.5 and 4.5.6.

3.12.4 FINAL IDENTIFICATION OF AEROBIC (AND FACULTATIVE) CLINICAL ISOLATES

The final identifications of aerobic and facultative isolates achieved by a combination of biochemical tests, commercial identification kits and partial 16S rRNA gene sequencing are given in tables 3.7; facultative species, 3.8; obligately aerobic species, 3.9; Capnophilic species. A total of 53 isolates were in this group of which 11 (**1** *1E*, **1** *1F*, **2** *1F*, **3** *1B*, **3** *2B*, **4** *2G*, **5** *1A*, **6** *2A*, **8** *2A*, **9** *2F* and **11** *1B*) ceased to be viable before identification could be completed (table 6.18, appendix 6.18) and an additional 3 (**1** *1A*, **4** *1C* I and **7** *2F*) were unidentified for reasons given in section 3.13.3.3 (table 3.10). Satisfactory identifications were obtained for 39 isolates, comprising 8 obligate aerobes, 28 facultative and 3 capnophilic isolates.

3.12.4.1 Successful identification by commercial identification kit

3.12.4.1.1 Identification by API NH alone

Four isolates (**4** *2A*, **5** *1C*, **6** *1A* and **7** *1B*) staining as Gram-negative cocci were identified as *Neisseria* species using API NH with an identity level of 97.5 - 99.8%. Using the test results given in table 3.5 and Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) these identifications were checked. The 'second stage table for the identification of *Acinetobacter*, *Branhamella*, *Gemella*, *Neisseria* and *Veillonella*' was used. *Neisseria* species usually display oxidative carbohydrate breakdown, with the exception of *N. cinerea* and *N. elongata* which show no action, other strains have a variable response to this test. Positive catalase and oxidase tests are also seen and there is an absence of growth under anaerobic conditions. Isolate **5** *1C* displays unusual fermentative action but is positive for oxidase and catalase and does not grow in an anaerobic environment. The production of yellow-brown pigment and lack of nitrate reduction by this isolate suggests *N. subflava* including *N. sicca, N. perflava* and *N. flava,* or *N. flavescens.* Isolate **6** *1A* gave a negative catalase test result as seen by *N. elongata* subsp. *elongata* and tested positive for growth in an anaerobic environment, although this is not common, enhanced growth of *Neisseria* species is seen in an atmosphere with increased CO_2 concentration. The yellow-brown colonies suggest *N. subflava*, *N. mucosa* or *N. lactimica* but the lack of nitrate reduction rules out *N. mucosa.* This conflict of speciation meant that this isolate was identified as '*Neisseria* species'. Isolate **7** *1B* tested negative for catalase and oxidase and positive for growth under anaerobic conditions; it also failed to reduce nitrates. The profile was nearer to that of *Gemella haemolysans* (previously called

Neisseria haemolysans), a Gram-positive coccus which is easily decolourised during the Gram stain and identified as Gram-negative. Isolate **4** *2A* was identified as *Neisseria cinerea* and showed the correct Gram morphology, despite the loss of viability of this isolate preventing confirmation of the species, the identification was accepted.

3.12.4.1.2 Identification by RapID ANA II System alone

See section 4.9.4 for information regarding the use of this kit to identify aerobic (and facultative or microaerophilic isolates).

Three Gram-negative bacilli (**2** *1A*, **8** *1A* and **10** *1A*) were identified as *Capnocytophaga* spp. with identity confidence levels of 99.9%. All three isolates died before they could be tested for their ability to grow anaerobically, but the identifications were accepted on the strength of the Gram morphology. Isolate **2** *1B* was identified as *Mobiluncus* sp. using RapID ANA II system (section 3.12.3.3).

3.12.4.1.3 Identification by Rapid ID 32 Strep alone

The fourteen Gram-positive cocci (**1** *1C*, **2** *1C*, **2** *1D*, **2** *2A*, **3** *1A*, **3** *1D*, **3** *1F*, **4** *1B*, **4** *1F*, **6** *2B*, **8** *1B*, **9** *1B*, **11** *2A* and **12** *1A*) identified using the Rapid ID 32 Strep identification kit were checked using the test results given in table 3.5 and Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993). All isolates fell into the genera *Streptococcus* or *Gemella*, matching with the identification provided by the identification kit. In all cases a species level identification was made including *S. constellatus*, *S. sanguis*, *S. mitis* and *Gemella haemolysans*. One isolate **9** *1B* was tested with a Rapid ID 32 Strep kit resulting in an identity level of 79.9% for *S. oralis*, this identification was accepted and confirmed by negative catalase and oxidase tests and the ability to grow anaerobically. Isolate **4** *1F* exhibited no EPS production on TYC agar. The result given by Rapid ID 32 Strep kit gave 81.9% identity *S. salivarius* subsp. *salivarius* which was above the threshold for acceptance. API suggested that 95% of *S. salivarius* isolates produce levan compared to only 1% of *Gemella morbillorum* isolates. Despite the negative EPS production of this isolate, the identification was accepted.

A region of the 16S rRNA gene of isolate **11** *2A* was sequenced and the isolate re-identified from *S. constellatus* to *S. anginosus*. This species was the second choice identification of the Rapid ID 32 Strep kit used to test this isolate. The identification of isolate **6** *2B* by

Rapid ID 32 Strep was also checked by sequencing, leading to the identification of this isolate being altered from *Lactococcus lactis cremoris* to the '*S. milleri*' group.

3.12.4.2 Identification of those isolates with high identification levels by commercial identification kit which was questioned

The identifications achieved for 11 isolates (see section 3.12.1) which gave good (above 79.9%) level identifications with commercial identification kits were rejected when subsequent tests (section 3.12.2) were considered and only 5 were available for sequence analysis (section 3.12.3), due to the death of the other isolates. Isolate **1** *1A* was identified as *Clostridium difficile* (using RapID ANA II - see section 4.9.4) and the Gram stain, negative catalase test and the lack of fermentative breakdown of carbohydrates agreed with the identification. However, it was discounted due to the aerobic cultivation of this isolate and the absence of oval central or subterminal spores. An identification of *Peptostreptococcus micros* was obtained for isolate 7 *2F*, however, the Gram morphology did not support this. Both isolates **1** *1A* and **7** *2F* were unidentified as explained in section 3.12.3.3. Isolates **9** *2A* and **14** *2A* were also identified as *N. cinerea* and demonstrated the correct Gram morphology. This identification was questioned due to the positive growth of both isolates under anaerobic conditions (although some species will grow in enhanced CO_2) and the negative catalase test of **14** *2A*. These two isolates were identified as *N. flavescens* by partial 16S rRNA gene sequencing and this was accepted due to the yellow-orange colonies characteristic of this species, formed on blood agar. Of the two isolates **4** *1C*, number II was identified as *Leuconostoc* sp. Despite the correlating Gram stain morphology, this identification was disregarded due to the lack of an identification of **4** *1C* I. Partial 16S rRNA gene sequencing resulted in an identification of *Stomatococcus mucilaginosus* for **4** *1C* **II**. An identification of isolate **4** *1C* **I** was not obtained for reasons described in section 3.12.3.3.

Isolate **6** *2A* obtained a 99.6% identity level to *Leuconostoc* sp., however the rod-shaped cells and lack of further information lead to this identification being rejected.

Isolate **4** *1A* was identified as *Neisseria cinerea*, an identification supported by the lack of nitrate reduction. *Neisseria* spp. are sometimes seen as coccobacilli therefore the identification was accepted when small bacilli were seen by Gram stain.

3.12.4.3 Identification of those isolates with low or absent identification levels by commercial identification kit which was not accepted

Of the 12 isolates which were unidentified by commercial identification kits (below 79.9% identity level, see section 3.12.1), 3 were unidentified, 6 were identified by partial 16S rRNA gene sequencing (**1** *1D*, **3** *1C*, **3** *2A*, **5** *2A*, **10** *2A* and **16** *1A*) and 3 had the original kit identification accepted. The profile of isolate **16** *1A* matched *Kingella*, and an identification of *K. dentrificans* was achieved by partial 16S rRNA gene sequencing. This was accepted despite the fact that most species ferment carbohydrate and reduce nitrates and this isolate displayed neither of these properties. Four isolates (**1** *1D*, **3** *1C*, **5** *2A* and **10** *2A*) placed into the genera *Streptococcus*, *Leuconostoc* or *Gemella* were all confirmed as *S. sanguis* (except **5** *2A*; *S. anginosus*) by partial 16S rRNA gene sequencing. Isolate **3** *2A* was identified as *N. elongata* by partial 16S rRNA gene sequencing and the traditional tests including a negative catalase test agree with this identification. This identification was accepted despite the bacilli seen in the Gram stain.

The identifications obtained by Rapid ID 32 Strep for three isolates (**9** *2B*, **13** *1A* and **14** *1A*) was accepted to genus level on the strength of Gram morphology and characteristics.

Eight isolates had no profile using identification kits (see section 3.12.1) of which 4 were subjected to sequence analysis. Adequate identifications of **1** *2B*, **3** *1E* and **9** *2E* were obtained. The Gram morphology of isolates **1** *2B* and **9** *2E* did not agree with the identification obtained by 16S rRNA gene sequencing. Isolate **1** *2B* and **9** *2E*, both Gram-positive rods were identified as Gram-positive cocci *S. gordonii* and *Leuconostoc mesenteroides* respectively. Traditional laboratory tests back-up the sequencing identifications which were therefore accepted. No identification was achieved for isolate **4** *1C* **I** as described in sections 3.12.3.3 and 3.12.4.2 above.

3.12.4.4 Identification of those isolates not identified by commercial identification kit

Seven isolates (**1** *1F*, **1** *2B*, **3** *1E*, **4** *1C* I, 9 ***2E***, **9** *2F* and **11** *1B*) gave no result when tested by commercial identification kit. Four isolates were examined by sequencing as described in section 3.12.3 and the remaining three (**1** *1F*, **9** *2F* and **11** *1B*) were not viable.

3.12.4.5 Unidentified isolates

Fourteen isolates were unidentified. Three (**1** *1A*, **4** *1C* I and **7** *2F*) due to contamination problems as described in section 3.12.3.3 and the remaining 11 isolates (**1** *1E*, **1** *1F*, **2** *1F*, **3**

1B, **3** *2B*, **4** *2G*, **5** *1A*, **6** *2A*, **8** *2A*, **9** *2F* and **11** *1B*) due to loss of viability. Isolates **3** *1B* and **5** *1A* were identified as *Cl. hastiforme* and *Cl. linosum* but had been isolated from aerobic culture, both isolates were lost before tests could be concluded and no identification was reached. Isolates **4** *2G* and **8** *2A* were identified as *Actinomyces meyeri*. **4** *2G* demonstrated the correct Gram morphology but was cultured solely in an aerobic environment, therefore the identification was not accepted. The identification of **8** *2A* was discounted on the basis of Gram stain, although it is conceivable that the Gram-negative result was a consequence of culture age, however the loss of this isolate prevented confirmation of this.

Isolate **6** *2A* remained unidentified as it was impossible to repeat the Gram stain.

FINAL IDENTIFCATIONS OF AEROBIC AND FACULATATIVE ISOLATES FROM PLS PATIENTS (TABLES 3.7-3.9)

Table 3.7 Facultative species isolated from the oral cavity of PLS patients

CLINICAL ISOLATE REFERENCE	FINAL IDENTIFICATION
1 *1C*	*S. bovis* II
1 *1D*	*S. sanguis*
1 *2B*	*S. gordonii*
2 *1B*	*Mobiluncus* sp.
2 *1C*	*S. constellatus*
2 *1D*	*G. morbillorum*
2 *2A*	*S. salivarius* subsp. *salivarius*
3 *1A*	*S. mitis*
3 *1C*	*S. sanguis*
3 *1D*	*G. haemolysans*
3 *1E*	*K. indologenes (S. indologenes)*
3 *1F*	*S. mitis*
4 *1B*	*S. constellatus*
4 *1C* II	*Stom. mucilaginosus*
4 *1F*	*S. salivarius subsp. salivarius*
5 *2A*	*S. anginosus*
6 *1B*	*Streptococcus* spp; '*S. milleri* group'
6 *2B*	'*S. milleri* group'
8 *1B*	*S. sanguis*
9 *1B*	*S. oralis*
9 *2B*	*S. mitis*
9 *2E*	*L. mesenteroides / L. cremoris*
10 *2A*	*S. sanguis*
11 *2A*	*S. anginosus*
12 *1A*	*S. constellatus*
13 *1A*	*Streptococcus* spp.
14 *1A*	*Streptococcus* spp.
16 *1A*	*K. denitrificans*

Table 3.8 Obligate aerobes isolated from the oral cavity of PLS patients

CLINICAL ISOLATE REFERENCE	IDENTIFICATION
3 *2A*	*N. elongata*
4 *1A*	*N. cinerea*
4 *2A*	*N. cinerea*
5 *1C*	*Neisseria* sp. possibly *N. subflava*
6 *1A*	*Neisseria* sp.
7 *1B*	*Neisseria* sp.
9 *2A*	*N. flavescens*
14 *2A*	*N. flavescens*

Table 3.9 Capnophilic species isolated from the oral cavity of PLS patients

CLINICAL ISOLATE REFERENCE	IDENTIFICATION
2 *1A*	*Capnocytophaga* sp.
8 *1A*	*Capnocytophaga* sp.
10 *1A*	*Capnocytophaga* sp.

Table 3.10 Unidentified species, for reasons other than loss of viability

CLINICAL ISOLATE REFERENCE	IDENTIFICATION
1 *1A*	No identification obtained
4 *1C* I	No identification obtained
7 *2F*	No identification obtained

KEY:

G., Gemella; K., Kingella; L., Leuconostoc; N., Neisseria; S., Streptococcus; Stom., Stomatococcus

3.13 IDENTIFICATION OF ANAEROBIC (AND FACULTATIVE) MICROORGANISMS ISOLATES FROM PATIENTS WITH PLS

3.13.1 COMMERCIAL IDENTIFICATION KITS

Thirty-eight isolates were labelled anaerobic or facultative. Seven isolates (**3** *1B*, **3** *1E*, **3** *2B*, **4** *2B*, 7 *1A*, 7 *1B* and **11** *2A*) ceased to be viable before identification could commence and were not tested by identification kit. Table 3.11 lists results obtained using commercial identification kits (Rapid ID 32 A and RapID ANA II system) to test 31 anaerobic and facultative isolates and does not necessarily represent final identifications which take into account other tests described in sections 3.13.2.1-3.13.2.4. Ten out of 31 isolates were tested with more than one identification kit. A total of 12 isolates (**1** *2B*, **2** *1B*, **2** *2A*, **3** *1D*, **3** *2A*, **5** *1D*, **5** *2B*, **6** *2A*, **8** *1A*, **17** *2A*, **18** *1D* and **18** *2A*) were successfully identified by commercial identification kits alone. Eight isolates were identified by Rapid ID 32A, seven (**1** *2B*, **3** *2A*, **5** ***1D***, **5** *2B*, **6** *2A*, **8** *1A*, **17** *2A*) were identified to species level; *P. intermedia* and *P. melaninogenica* using Rapid ID 32 A with percent identity levels ranging from 74.0% to 99.9% with the exception of **8** *1A*. An additional isolate (**18** *1D*) was identified as *Gemella morbillorum*. A positive genus level identification was achieved were as identity of 79.0% or more was seen, with the occasional exception (e.g. isolate **8** *1A*). Using the RapID ANA II system, the identity of 3 isolates (**1** *2B*, **3** *2A* and **5** *1D*) was confirmed and a further 3 isolates (**2** *1B*, **3** *1D* and **18** *2A*) were identified as *Mobiluncus* species and peptostreptococci. One isolate (**2** *2A*) was identified using Rapid 32 Strep as *Leuconostoc* sp. An identity level of 75.6% was accepted in this case and confirmed by the Gram morphology (3.13.2.1) and other tests (3.13.2.2 - 3.13.2.4). These isolates are marked in table 3.11 with symbols which cross-reference individual test results which can be found tables 6.15, 6.16 and 6.17 in appendix 6.17.2.

Of the remaining 19 isolates, 8 (**1** *1B*, **3** *1A*, **4** *1E*, **4** *2A*, **10** *1B*, **10** *2A*, **10** *2B* and **12** *2A*) gave good (above 79.9%) level identifications with commercial identification kits. Six of these isolates (except **4** *1E* and **10** *2A*) were tested further. All of these identifications were rejected later on the basis of further tests described in sections 3.13.2.1-3.13.2.4.

Table 3.11 List of anaerobic (and facultative) clinical isolates and the commercial kits used for identification

A key to symbols and abbreviations is provided at the end of the table.

CLINICAL ISOLATE	BIOCHEMICAL KIT(S) USED	RESULTS	% ID	LEVEL
1 *1B*	RAPID ANA II	*E. aerofaciens*	79.74	Probability overlap.
		Bifidobacterium	14.83	Level inadequate
		Actinomyces israelii	3.79	
	RAPID ID 32 A	Unacceptable profile	—	—
1 *2A*	RAPID ANA II	*Prop. granulosum*	62.56	Probability overlap
		Mobiluncus sp.	34.44	
1 *2B*	RAPID ANA II	*P. intermedia*	99.99	Satisfactory ⊗
	RAPID ID 32 A	*P. intermedia*	99.9	Excellent ID ⊕
2 *1B*	RAPID ANAII	*Mobiluncus* sp.	99.9	Satisfactory ⊗
2 *2A*	RAPID ID 32 STREP†	*Leuconostoc* sp.	75.6	Doubtful profile
		Lc. lactis cremoris	24.0	
2 *2B*	RAPID ANA II	Questionable code	—	—
3 *1A*	RAPID ANA II	*Mobiluncus* sp.	85.69	Probability overlap
		Clostridium botulinum	10.92	
	RAPID ANA II	*Bifidobacterium*	68.88	Probability overlap
		A. israelii	26.72	
3 *1D*	RAPID ANA II	*Peptostreptococcus micros*	98.80	Satisfactory ⊗
3 *2A*	RAPID ID 32 A	*P. intermedia*	99.9	Excellent ID ⊕
	RAPID ANA II	*P. intermedia*	99.9	Implicit ⊗
4 *1E*	RAPID ID 32 A	*A. meyeri*	95.3	Doubtful profile
4 *2A*	RAPID ANA II	*Cl. sporogenes*	85.7	—
		Cl. difficile	14.09	
5 *1A*	RAPID ANA II	*Bifidobacterium*	68.88	Probability overlap
		A. israelii	26.72	
	RAPID ANA II	*Cl. sporogenes*	85.75	Probability overlap
		Cl. difficile	10.49	
5 *1B*	RAPID ID 32 A	*Bif. adolescentis*	39.6	Doubtful profile
		A. naeslundii	28.8	
		A. viscosus	18.6	
		Prop. propionicum	10.9	
5 *1D*	RAPID ID 32 A	*P. intermedia*	99.5	Very good ID ⊕
	RAPID ANA II	*P. intermedia*	99.9	Implicit ⊗
5 *2B*	RAPID ID 32 A	*P. melaninogenica*	98.6	Good ID ⊕

CLINICAL ISOLATE	BIOCHEMICAL KIT(S) USED	RESULTS	% ID	LEVEL
6 *1A*	RAPID ANA II	Questionable code	—	—
	RAPID ID 32 A	Unacceptable profile	—	—
6 *2A*	RAPID ID 32 A	*P. melaninogenica*	86.8	Acceptable ID ⊕
		P. denticola	11.1	
7 *2B*	RAPID ANA II	Unacceptable code	—	—
	RAPID ID 32 A	Unacceptable profile	—	—
8 *1A*	RAPID ID 32 A	*P. oralis*	74.0	Very good ID to genus ⊕
		P. denticola	18.7	
		P. melaninogenica	5.9	
		P. bivia	1.2	
10 *1A*	RAPID ID 32 A	Unacceptable profile	—	—
10 *1B*	RAPID ID 32 A	*A. israelii*	99.2	Doubtful profile
	RAPID ANA II	Unacceptable code	—	—
10 *2A*	RAPID ID 32 A	*Cl. sordellii*	92.4	Good ID
		F. nucleatum	3.8	
10 *2B*	RAPID ID 32 A	*Pstr. micros*	92.9	Doubtful profile
		A. meyeri	4.1	
11 *1A*	RAPID ANA II	Unacceptable profile	—	—
12 *2A*	RAPID ANA II	Unacceptable code	—	—
	RAPID ID 32 A	*A. naeslundii*	99.7	Good ID
13 *2A*	RAPID ANA II	Unacceptable code	—	—
	RAPID ID 32 A	*Pstr. anaerobius*	41.1	Doubtful profile
		Eubacterium limosum	26.7	
		Cl. difficilie	13.3	
		Bac. ureolyticus	7.5	
14 *2E*	RAPID ID 32 A	*Cl. difficile*	66.0	Low discrimination
		Cl. bifermentans	24.6	
		Bac. ureolyticus	5.2	
15 *1A*	RAPID ID 32 A	Unacceptable profile	—	—
	RAPID ANA II	Questionable code	—	—
17 *2A*	RAPID ID 32 A	*P. melaninogenica*	89.4	Acceptable ID ⊕
18 *2A*	RAPID ANA II	*Pstr. anaerobius*	98.56	Satisfactory ⊗
18 *1D*	RAPID ID 32 A	*Gemella morbillorum*	99.9	Very good ID ⊕

KEY:

A., Actinomyces; Bif., Bifidobacterium; Bac., Bacteroides; Cl., Clostridium; F., Fusobacterium; Lc., Lactococcus; P., Prevotella; Prop., Propionibacterium; Pstr., Peptostreptococcus

⊕ See table 6.15 (appendix 6.17.2) for individual test results from Rapid ID 32 A.

⊗ See table 6.16 (appendix 6.17.2) for individual test results from RapID ANA II system.

† Many streptococcal species are facultative, therefore the Rapid ID 32 Strep kit was used for Gram-positive coocci growing anaerobically.

FOOTNOTES TO TABLE 3.11.

1. Gram morphologies are listed in table 3.12.
2. A description of rationale behind choice of kit is provided in material and methods section 2.10.1 and discussed in sections 4.9.4 and 4.10.1.3 of the discussion.
3. The identification achieved is given but may not represent final identifications, which take into account Gram stain morphology (table 3.12), other tests (table 3.13), GLC (table 3.14) and partial 16S rRNA gene sequencing (table 3.15). Final identifications are presented in tables 3.16 and 3.17.

A further 5 isolates (**1** *2A*, **5** *1A*, **5** *1B*, **13** *2A* and **14** *2E*) gave low level identifications (below 79.0%) which were not accepted, and all except **5** *1B* were tested further as described in sections 3.8.2.1-3.8.2.4. Six isolates (**2** *2B*, **6** *1A*, **7** *2B*, **10** *1A*, **11** *1A* and **15** *1A*) gave no result (labelled acceptable profile or questionable code in table 3.11) with one or more identification kits all were subjected to subsequent tests.

3.13.2 SUBSEQUENT IDENTIFICATION

3.13.2.1 Gram stain and cellular morphology

Table 3.12 lists the Gram stain results for all 38 anaerobic and facultative isolates, this includes 3 isolates (**3** *1B*, **4** *1E* and **11** *2A*) for which the Gram morphology was not obtained or completed and could not be tested due to the loss of viability of these isolates. A complete list of isolates which ceased to be viable before identification could be completed is found in appendix 6.18 in table 6.18. The Gram morphology of 1 isolate (**10** *1B*) was not provided by MRI and in 3 cases the Gram result was different from that provided by MRI (**1** *2B* - GPB; **10** *2B* - GNB; **13** *2A* - GNC). A total of 23 were Gram-positive, of which 9 were cocci and 14 bacilli. Of the 13 Gram-negative isolates, 12 were bacilli and 1 was undetermined. These results were used in combination with those obtained by commercial identification kits to accept or disprove identifications over 79.0%.

3.13.2.2 Catalase and oxidase activity, growth in an aerobic environment and colony colour

The results of catalase and oxidase tests, the ability of isolates to grow in an aerobic environment and colony colour are recorded in table 3.13. These characteristics were recorded in 28 cases and included one isolate which had already been identified (**17** *2A*).

Colony colour was recorded in only 15 cases (**1** *2A*, **1** *2B*, **2** *2A*, **2** *2B*, **3** *1D*, **3** *2A*, **5** *1D*, **5** *2B*, **6** *1A*, **6** *2A*, **8** *1A*, **10** *1B*, **11** *1A*, **17** *2A* and **18** *1D*) and is also shown in table 3.13. These included 5 yellow-pink isolates and 6 brown-black pigmenting isolates. These results were used in combination with the Gram stain results and the identification tables given in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) to provide a genus level identification and to assess the results obtained by commercial identification kits.

Table 3.12 Gram stain morphologies for anaerobic and facultative clinical isolates

These results represent those achieved during this study, the majority of which correspond to those received from staff at MRI.

CLINICAL ISOLATE REFERENCES	GRAM STAIN RESULT
1 *1B*	GPB
1 *2A*	GPB
1 *2B*	GNB §
2 *1B*	GPB
2 *2A*	GPC
2 *2B*	GPC
3 *1A*	GPB
3 *1B*	Unknown
3 *1D*	GPC
3 *1E*	GPB
3 *2A*	GNB
3 *2B*	GNB
4 *1E*	GN †
4 *2A*	GPB
4 *2B*	GNB
5 *1A*	GPB
5 *1B*	GPB
5 *1D*	GNB
5 *2B*	GNB

CLINICAL ISOLATE REFERENCE	GRAM STAIN RESULT
6 *1A*	GPC
6 *2A*	GNB
7 *1A*	GNB
7 *1B*	GNB
7 *2B*	GPB
8 *1A*	GNB
10 *1A*	GPB
10 *1B*	GPB ‡
10 *2A*	GNB
10 *2B*	GPB §
11 *1A*	GPC
11 *2A*	Unknown
12 *2A*	GPC
13 *2A*	GPC §
14 *2E*	GNB
15 *1A*	GPB
17 *2A*	GNB
18 *2A*	GPC
18 1*D*	GPC

KEY:

GPB, Gram-positive bacilli; GNB, Gram-negative bacilli; GPC, Gram-positive cocci; GNC, Gram-negative cocci

† Cell shape not provided by MRI, isolate died before it could be tested.

§ Gram result different from MRI (**1** *2B* - GPB; **10** *2B* - GNB; **13** *2A* - GNC).

‡ Gram morphology not supplied by MRI.

Table 3.13 Results of catalase and oxidase tests, ability to grow in an aerobic environment and colony colour for each anaerobic and facultative isolate

CLINICAL ISOLATE REFERENCE	CATALASE TEST	OXIDASE TEST	GROWTH IN AEROBIC ENVIRONMENT	COLONY COLOUR
1 *1B*	-	-	-	NT
1 *2A*	-	-	-	P-Y
1 *2B*	-	-	-	Bl
2 *1B*	-	NT	-	NT
2 *2A*	-	-	+	W
2 *2B*	-	-	+	P-Y
3 *1A*	NT	-	NT	NT
3 *1D*	-	-	-	S-G
3 *2A*	-	-	-	Bl
4 *2A*	+	-	-	NT
5 *1A*	-	-	-	NT
5 *1D*	-	-	-	Bl
5 *2B*	-	-	-	Br-B
6 *1A*	-	-	+	Y
6 *2A*	NT	NT	NT	Bl
7 *2B*	+	-	-	NT
8 *1A*	NT	NT	NT	Bl
10 *1A*	+	-	-	NT
10 *1B*	+	-	-	P
10 *2B*	-	+	+	NT
11 *1A*	-	-	-	Y
12 *2A*	-	-	+	NT
13 *2A*	+	-	+	NT
14 *2E*	-	+	-	NT
15 *1A*	-	-	+	NT
17 *2A*	NT	NT	NT	Bl
18 1*D*	-	-	+	W
18 *2A*	-	-	-	NT

KEY:

C, cream; B, beige; Bl, black; Br, brown; G, grey; NT, not tested; P, pink; Or, orange; S, silver; W, white; Y, yellow

3.13.2.3 Spore stain

Isolates identified as Gram-positive bacilli (**4** *2A*, **10** *1A*, **10** *1B* and *14* **2E**) were stained for spore formation. All spore stains were negative when a 5 day old culture was tested.

3.13.2.4 Gas-Liquid Chromatography

Twenty-five isolates were analysed by Gas-Liquid chromatography (GLC), they were grown in GLC broth (appendix 6.1.4.3) and the culture supernatant analysed for the production of volatile acids and alcohols. Only Gram-positive isolates and Gram-negative isolates producing no volatile end-products, were analysed for the production of non-volatile acids, this excluded four isolates (**1** *2B*, **3** *2A*, **5** *1D* and **5** *2B*).

The retention times of peaks were compared to those of known standards analysed under identical conditions (the range of time, in minutes, over which substances were eluted and the median values are given in tables 6.19 and 6.20 in appendix 6.19). The height of the peaks recorded on the chart recorder was compared to the height produced by culture medium alone and the production was only recorded as positive if the test peak was higher. GLC results for both volatile and non-volatile substances with suggested genus level identifications, are listed in table 3.14. Results were used to determine genus level identifications according to the table given in the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) and adapted for to illustrate its use for oral anaerobes in appendix 6.20, table 6.21. GLC results were compared to those results achieved by other means (sections 3.13.1 and 3.13.2.1-3.13.2.3) and considered when formulating the final identification.

3.13.2.4.1 Analysis of volatile acids and alcohols

Ten isolates (**1** *2A*, **2** *1B*, **2** *2B*, **3** *1A*, **4** *2A*, **6** *1A*, **10** *1A*, **10** *2B*, **14** *2E* and **18** *2A*) produced no volatile acids or alcohols. Seven isolates tested (**1** *2B*, **3** *1D*, **3** *2A*, **5** *1A*, **5** *1D*, **5** *2B* and **11** *1A*) had major acetic acid peaks and in two cases (**3** *1D* and **5** *1A*) it was the sole major product. Eight isolates (**1** *1B*, **2** *2A*, **7** *2B*, **10** *1B*, **12** *2A*, **13** *2A*, **15** *1A* and **18** *1D*) had no major product but minor peaks of acetic acid, propanol or butanol were seen. Propionic, valeric and *iso*-caproic acids were produced by only one isolate (**11** *1A*). *Iso*-butyric acid and *iso*-valeric acid were major products for 5 isolates (**1** *2B*, **3** *2A*, **5** *1D*, **5** *2B* and **11** *1A*).

The order of elution and the retention time of the solute was established using volatile-acid and alcohol standards as described in the materials and methods section 2.11.3.2.5. A

typical chromatogram produced by the running of volatile acid and alcohol standards is seen in figure 3.17.

All cultures were grown in GLC broth (Lab M) which therefore constituted the culture supernatant. The presence of volatile substances within the GLC broth was tested by treating uncontaminated broth as described in section 2.11.3.2.3 and injecting it onto the column (see section 2.11.3.2.4). The chromatogram seen in figure 3.18 demonstrates that the GLC broth contained only trace amounts of acetic acid. The presence of acetic acid was only recorded if the height of the test peak was larger than that seen when GLC broth was tested. The traces of the 5 isolates (**1** *2B*, **3** *2A*, **5** *1D*, **5** *2B* and **11** *1A*) recording iso-butyric and iso-valeric acids as the major product is demonstrated in figure 3.19 for isolate **3** *2A*. One isolate (**11** *1A*) produced propionic, valeric and *iso*-caproic acids and this GLC chromatogram is shown in figure 3.20. The identity of the peaks was confirmed by loading culture supernatant and standards simultaneously as shown in figure 3.21.

3.13.2.4.2 Analysis of non-volatile acids (as methyl esters)

Four isolates (**2** *2B*, **3** *1D*, **4** *2A* and **13** *2A*) produced no non-volatile acids. Lactic acid was the major non-volatile acid peak produced by 10 isolates (**1** *1B*, **1** *2A*, **2** *2A*, **3** *1A*, **5** *1A*, **6** *1A*, 7 *2B*, **12** *2A*, **15** *1A* and **18** *1D*); three isolates (**1** *1B*, **3** *1A* and **15** *1A*) had an additional major succinic acid peak while succinic acid was the sole major end product in 2 cases (**2** *1B* and **18** *2A*). Fumaric acid and malonic acid were detected in only two cases, viz. **10** *1A* and **15** *1A*. The order of elution and the retention time of non-volatile substances was established using non-volatile acid standards as described in the materials and methods section 2.11.3.3.4.

Non-volatile acid standards are illustrated in figure 3.22 and figure 3.23 indicates the presence of lactic and succinic acids as their methyl esters in the methylated extract of GLC broth. As with volatile acids, a peak height larger than that seen for broth alone was positive. A typical GLC trace of non-volatile acids from isolate **1** *1B* is seen in figure 3.24. Co-chromatography (Drucker, 1981) of sample and standards was used to test the identity of non-volatile substances as well as volatile ones. Figure 3.25 shows a GLC chromatogram from isolate **10** *1A* which contains a peak between lactic and succinic acids. Figure 3.26 shows that the presence of a fifth peak with the standards indicates that it does not represent malonic acid and is possibly fumaric acid which occurs between malonic and

succinic acids. Figure 3.27 shows the unusual chromatogram of isolate **11** *1A* which as an unidentified peak after succinic acid.

Table 3.14 and figures 3.17-3.27 are found on the following 12 pages, the key below applies to table 3.14 and all figures.

KEY:

2, Ethanol; **3,** Propanol; **4,** Butanol;

A, acetic acid, major peak or standard; **a,** acetic acid, minor peak;

P, propionic acid, major peak or standard; **p**, propionic acid, minor peak

B, butyric acid, major peak or standard; **b,** butyric acid, minor peak;

iB, *iso*-butyric acid, major peak or standard; **ib,** *iso*-butyric acid, minor peak;

iC, *iso*-caproic acid, major peak or standard; **ic**, *iso*-caproic acid, minor peak;

iV, *iso*-valeric acid, major peak or standard; **iv,** *iso*-valeric acid, minor peak;

V, valeric acid, major peak or standard; **v,** valeric acid, minor peak;

PY, pyruvate, major peak or standard;

L, lactic acid, major peak or standard; **l,** lactic acid, minor peak;

O, oxalic acid, major peak or standard; **o,** oxalic acid, minor peak;

S, succinic acid, major peak or standard; **s,** succinic acid, minor peak or standard;

M, malonic acid, major peak or standard; **m,** malonic acid, minor peak or standard

Table 3.14 Results of Gas-Liquid Chromatography analysis (volatile acids and alcohols and non-volatile acids) produced by anaerobic and facultative bacteria

CLINICAL ISOLATE REF	VOLATILE ACIDS AND ALCOHOLS	NON-VOLATILE ACIDS	GENUS LEVEL IDENTIFIATION SUGGESTED BY GLC	FINAL GENUS LEVEL IDENTIFICATION
1 *1B*	a	L, S	*Actinomyces*	*Actinomyces*
1 *2A*	-	L	*Eubacterium*	*Eubacterium*
1 *2B*	A, iB, iV	not tested	*Bacteroides / Prevotella / Porphyromonas / Bilophila*	*Prevotella*
2 *1B*	-	S	NG	*Mobiluncus*
2 *2A*	(3)	L, s	*Gemella / Leuconostoc / Streptococcus /*	*Leuconostoc*
2 *2B*	-	-	*Gemella / Leuconostoc / Streptococcus /*	*Streptococcus*
3 *1A*	-	L, S	*Actinomyces*	*Actinomyces*
3 *1D*	A	-	*Peptostreptococcus*[⊗]	*Peptostreptococcus*
3 *2A*	A, iB, iV	not tested	*Bacteroides / Prevotella / Porphyromonas / Bilophila*	*Prevotella*
4 *2A*	-	-	*Eubacterium*	NO ID
5 *1A*	A (4)	L, s	*Actinomyces*	NO ID
5 *1D*	A, iB, iV	not tested	*Bacteroides / Prevotella / Porphyromonas / Bilophila*	*Prevotella*
5 *2B*	A, iB, iV	not tested	*Bacteroides / Prevotella / Porphyromonas / Bilophila*	*Prevotella*
6 *1A*	-	L	*Gemella / Leuconostoc / Streptococcus /*	*Streptococcus*
7 *2B*	a, (3)	L, s	*Actinomyces*	No ID
10 *1A*	-	S, f	*Eubacterium*	No ID
10 *1B*	a	L, S	*Actinomyces*	No ID
10 *2B*	-	S	*Eubacterium*	No ID

CLINICAL ISOLATE REF	VOLATILE ACIDS AND ALCOHOLS	NON-VOLATILE ACIDS	GENUS LEVEL IDENTIFIATION SUGGESTED BY GLC	FINAL GENUS LEVEL IDENTIFICATION
11 *1A*	(3), A, iB, iV, iC, b, p	PY, l, m, s, other	*Peptostreptococcus* ⊗	*Peptostreptococcus*
12 *2A*	(3)	L, s	*Gemella* / *Leuconostoc* / *Streptococcus* /	*Streptococcus*
13 *2A*	a	-	*Eubacterium*	No ID
14 *2E*	-	S	*Bacteroides* / *Prevotella* / *Porphyromonas* / *Bilophila*	*Eikenella*
15 *1A*	(3)	L, S, m	NG	*Bacillus*
18 *1D*	(3)	L, s	*Gemella* / *Leuconostoc* / *Streptococcus* /	*Gemella*
18 *2A*	-	S	*Peptostreptococcus*⊗	*Peptostreptococcus*

KEY:

All abbreviations for metabolic end-products are described on page 214.

No ID, No identification.

NG, No genus level identification could be accepted for these GLC results.

⊗ Isolates did not produce any major peaks in GLC analysis or profile very different to that expected for *Gemella, Leuconostoc* or *Streptococcus*. Identification of *Peptostreptococcus* made accepted due to Gram stain (table 3.12), other tests (table 3.13) and the identification kit result (table 3.11).

FOOTNOTE TO TABLE 3.14.

1. Suggested genus level identifications from GLC results were made using the Gram stain result and the table for differentiation of genera of anaerobes table 5.1 found on pages 67-68 of the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993). An abridged version is showing the relevant organisms is found in appendix 6.20, table 6.21.
2. Gram stain results are shown in table 3.12.

Figure 3.17 Typical GLC chromatogram of volatile acid and alcohol standards

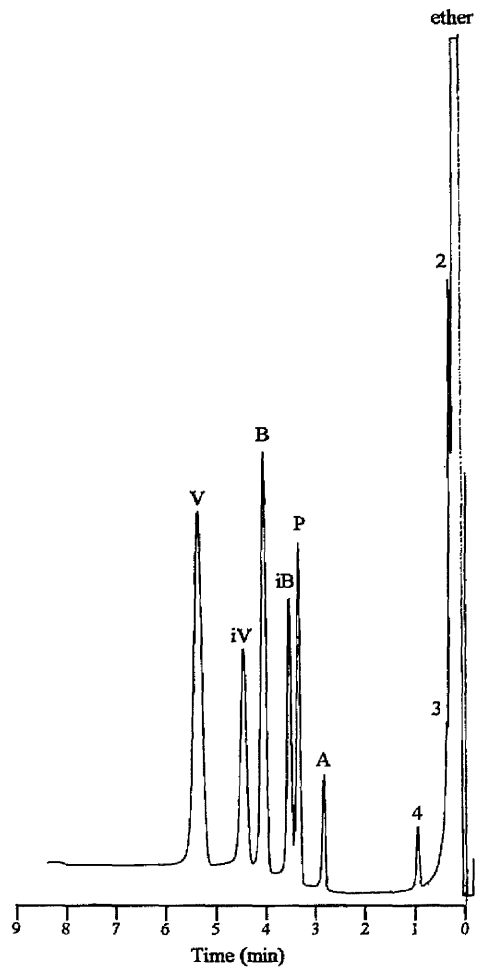


Figure 3.18 Typical GLC chromatogram of sterile acidified GLC broth

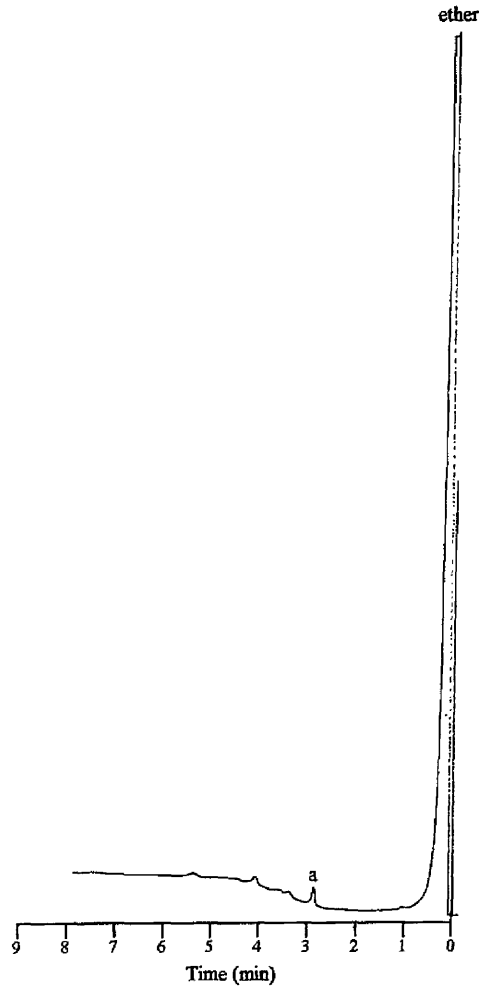


Figure 3.19 Typical GLC chromatogram of *Prevotella* spp. (isolate 3 *2A*)

Isolates **1** *2B*, **2** *3A*, **3** *2A*, **5** *1D* and **5** *2B* displayed a similar profile with major *iso*-butyric and *iso*-valeric acid peaks.

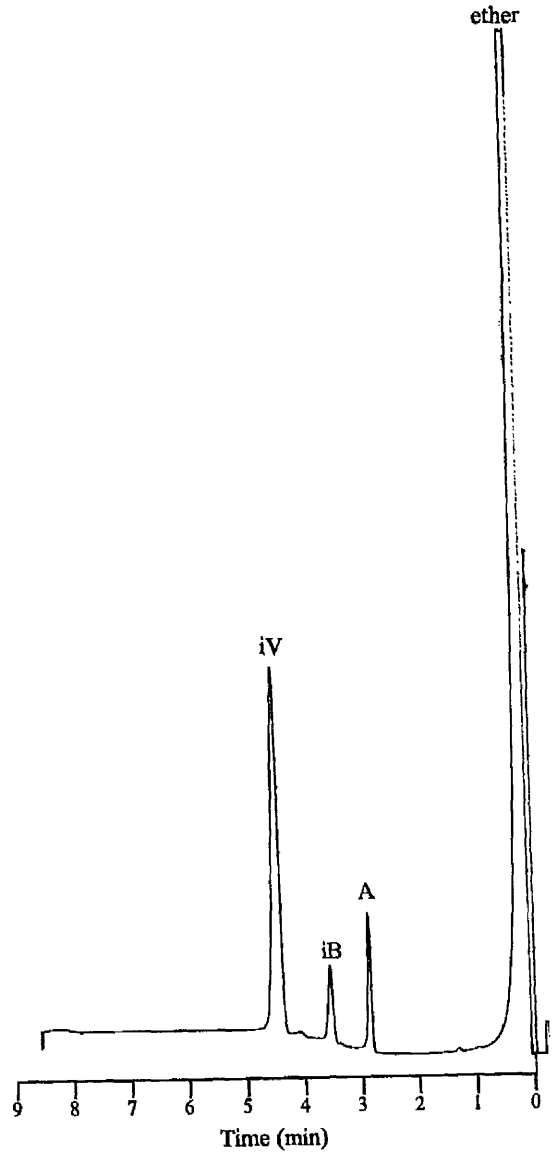


Figure 3.20 GLC chromatogram from isolate 11 *1A*, which produced *iso*-caproic acid

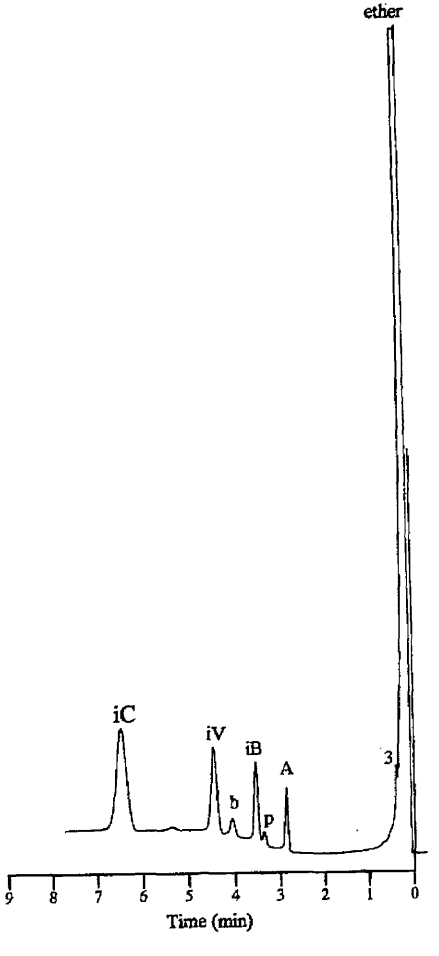


Figure 3.21 GLC chromatogram from isolate 11 *1A*, which produced *iso*-caproic acid

Ether extract of culture supernatant from isolate **11** *1A* was injected with volatile acid and alcohol standards to confirm the occurrence of an extra peak.

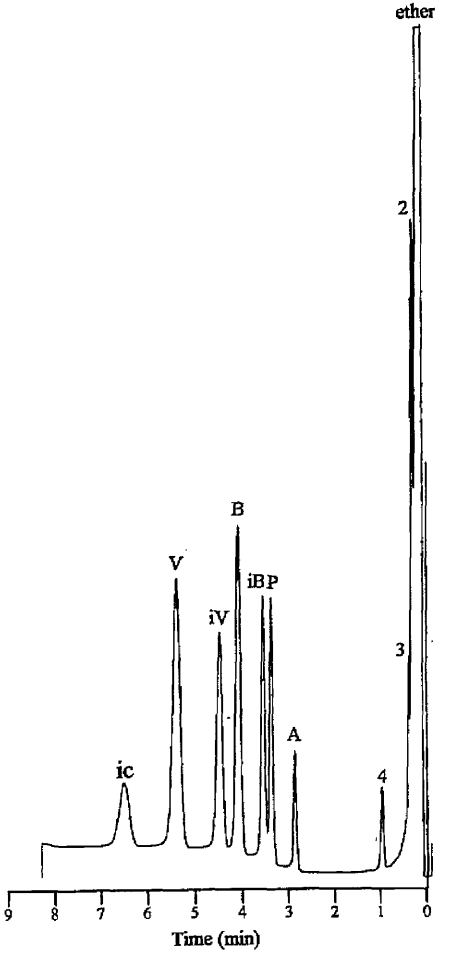


Figure 3.22 Typical GLC chromatogram of non-volatile acid standards (as methyl esters)

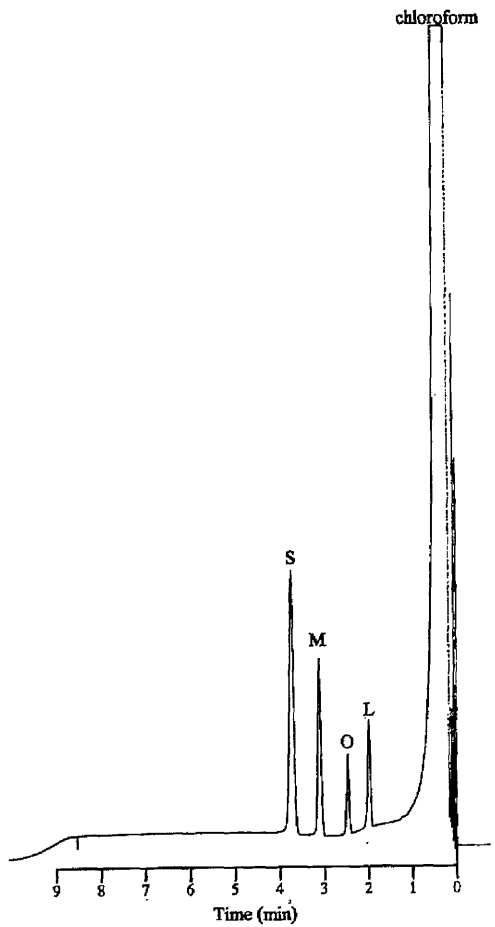


Figure 3.23 Typical GLC chromatogram of sterile methylated GLC broth

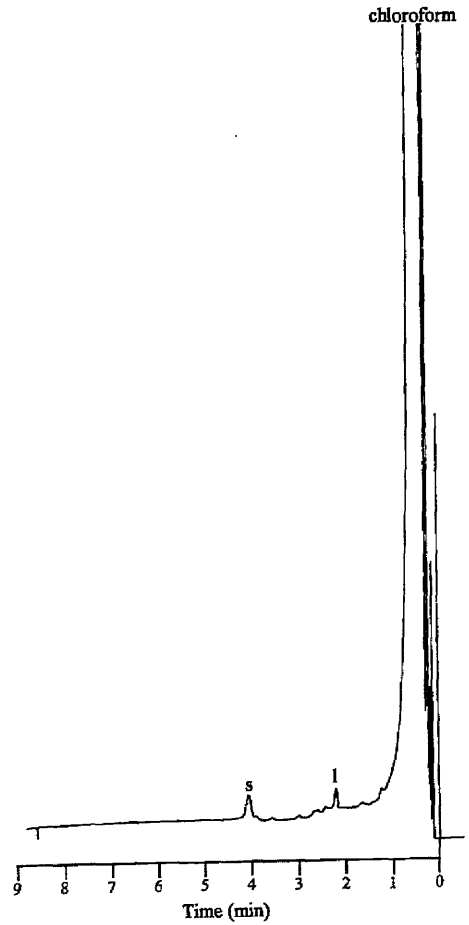


Figure 3.24 A typical GLC chromatogram of non-volatile acids from isolate 1 *1B*, analysed as their methyl esters

Figure 3.25 GLC chromatogram from isolate 10 *1A*

The GLC chromatogram from isolate **10** *1A* shows production of an extra peak between lactic and succinic acids; it is unclear whether it represents malonic acid.

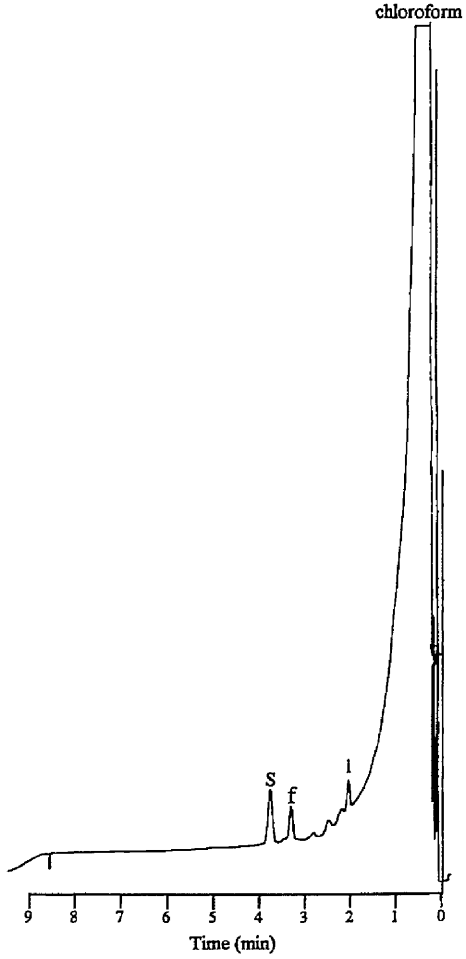


Figure 3.26 GLC chromatogram from isolate 10 *1A* plus non-volatile acid standards

Addition of non-volatile acid standards results in a fifth peak in addition to malonic acid showing that isolate **10** *1A* does not produce malonic acid.

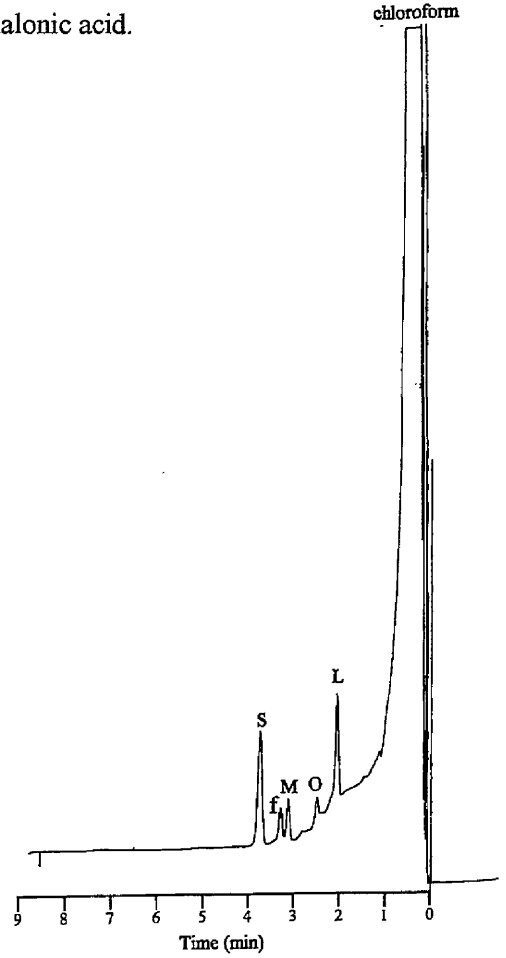
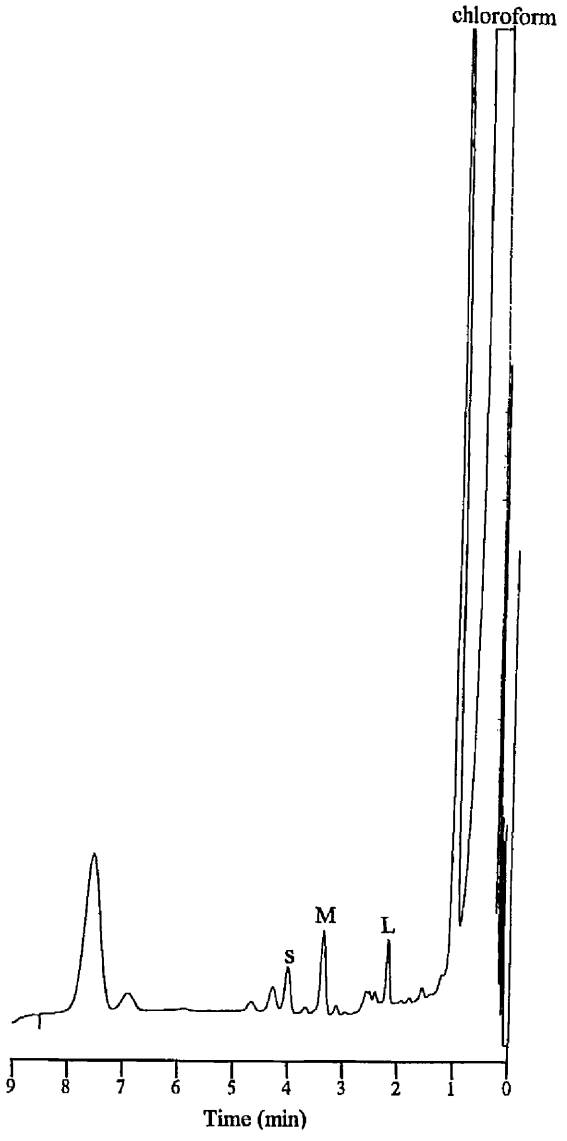


Figure 3.27 GLC chromatogram of non-volatile acids produced by isolate 11 *1A*, displaying an unidentified peak after succinic acid



3.13.3 PARTIAL SEQUENCING OF 16S RRNA GENE

3.13.3.1 Isolates which were sequenced

Eleven isolates (**1** *1B*, **1** *2B*, **3** *1A*, **3** *2A*, **5** *1A*, **5** *1D*, **6** *1A*, **7** *2B*, **11** *1A*, **12** *2A* and **15** *1A*) were subjected to DNA extraction and partial sequencing of the 16S rRNA gene. The three isolates identified as *P. intermedia* (**1** *2B*, **3** *2A* and **5** *1D*) were analysed by sequencing to determine whether any of them were actually *P. nigrescens*. Five isolates (**4** *1E*, **10** *1B*, **10** *2A*, **10** *2B* and **12** *2A*) gave good (above 79.9%) level identifications with commercial identification kits, which were discounted on the basis of Gram morphology and other tests, of these isolates, only 1 (**12** *2A*) was available for analysis by sequencing.

3.13.3.2 Suggested identifications of the isolates to be sequenced

The identification of isolate **12** *2A* as *A. naeslundii* was discounted due to the coccoid cells seen by Gram stain and a GLC profile suggestive of *Streptococcus* or *Gemella*.

Four isolates (**1** *1B*, **1** *2A*, **3** *1A* and **5** *1A*) which gave unacceptable low level identifications by commercial kits, were tested by this technique. Catalase, oxidase tests, Gram morphology and growth in an aerobic environment allowed genus identifications to be suggested for **1** *1B* and **5** *1A*. Gram morphology, GLC profile and other tests suggest an identification of *Bifidobacterium* or *Actinomyces* for isolate **5** *1A*. Isolate **3** *1A* may also fall into either of these two genera on the basis of the RapID ANA II system and GLC analysis but other tests are incomplete and cannot be used in this case. Isolate **1** *1B* was identified as *Eubacterium aerofaciens* with a level of 79.74%, the threshold for acceptance. This isolate was shown to be catalase- and oxidase-negative and demonstrated Gram morphology and growth characteristics that corroborated this identification. This identification was questioned and the organism was examined by partial 16S rRNA sequence analysis due to major lactic and succinic acid peaks seen with GLC analysis, more characteristic of *Actinomyces* species.

Seven isolates were unidentified by commercial kits. Genus level identifications were made for 5 of these isolates (**2** *2B*, **6** *1A*, **7** *2B*, **11** *1A* and **15** *1A*) based on the Gram morphology, GLC analysis and other tests. The identifications of 4 isolates as *Streptococcus* (*Gemella* or *Leuconostoc*) (**6** *1A*), *Peptostreptococcus* (**11** *1A*), *Propionibacterium* (**7** *2B*) and *Bacillus* sp. (**15** *1A*) was tested by partial 16S rRNA sequencing.

3.13.3.3 Analysis of sequence data

Partial 16S rDNA sequences were obtained for 13 anaerobic and facultative isolates. A FASTA search was used to provide the most likely phylogenetic relationship and this was checked against the results of the commercial identification kits (table 3.11) Gram stains (table 3.12) and other tests (table 3.13) described in sections 3.13.2.1 - 3.13.2.4. Table 3.15 compares the results obtained by commercial identification kits and partial 16S rRNA gene sequencing. In 3 cases (**1** *2B*, **3** *2A* and **5** *1D*), the genus level identification of *Prevotella* achieved by commercial identification kits and physiological tests matched that achieved by partial 16S rRNA gene sequencing. Of these 3 isolates identified as *P. intermedia*, **1** *2B* and **3** *2A* were identified as *P. nigrescens*. The sequence information of 1 isolate (**5** *1A*) was discarded due to the improbable identifications of *Pseudomonas*, thought to be the result of contamination. Sequence information is given in appendix 6.21.2.

3.13.4 FINAL IDENTIFICATION OF ANAEROBIC (AND FACULTATIVE) CLINICAL ISOLATES

The final identifications of anaerobic and facultative isolates achieved by a combination of biochemical tests, commercial identification kits, GLC analysis and partial 16S rRNA gene sequencing are given in tables 3.16; facultative isolates and 3.17; obligately anaerobic isolates. A total of 38 isolates were in this group of which 15 (**3** *1B*, **3** *1E*, **3** *2B*, **4** *1E*, **4** *2A*, **4** *2B*, **5** *1B*, 7 *1A*, 7 *1B*, **10** *1B*, **10** *2A*, **10** *2B*, **11** *2A*, **13** *2A* and **14** *2E*) were unidentified due to cell death, an additional 2 (**5** *1A*, **10** *1A*) was unidentified for reasons described in section 3.13.3.3 (table 3.18). Twenty-one satisfactory identifications were obtained comprising 11 obligate anaerobes and 10 facultative / microaerophilic isolates.

3.13.4.1 Successful identification by commercial identification kit

3.13.4.1.1 Identification by Rapid ID 32 A

Seven isolates staining as Gram-negative bacilli were identified as pigmented *Prevotella* species. The colony colour was recorded as brown to black. Three isolates (**6** *2A*, **8** *1A* and **17** *2A*) died before further tests could be conducted. The identifications were accepted but isolate **8** *1A* could not be speciated and was classified only as *Prevotella* spp. Isolates **5** *2B*, **6** *2A* and **17** *2A* were identified as *P. melaninogenica*. GLC analysis of isolate **5** *2B* placed this isolate within *Prevotella* or *Porphyromonas*. Three isolates (**1** *2B*, **3** *2A* and **5** *1D)* were identified as *P. intermedia* and confirmed using the RapID ANA II system.

Table 3.15 Comparison of identifications of anaerobic and facultative isolates obtained by commercial identification kits and partial 16S rDNA sequencing

CLINICAL ISOLATE REFERENCE	COMMERCIAL KIT IDENTIFCATION	PARTIAL 16S RRNA SEQUENCING IDENTIFICATION
1 *1B*	*E. aerofaciens*	*A. israelii*
1 *2B*	*P. intermedia*	*P. nigrescens*
3 *1A*	*Mobiluncus*	*Actinomyces* sp.
3 *2A*	*P. intermedia*	*P. nigrescens*
5 *1A*	*Bifidobacterium / A. israelii*	*Pseudomonas* sp.
5 *1D*	*P. intermedia*	*P. intermedia*
6 *1A*	No Identification	*S. sanguis*
7 *2B*	No Identification	*S. gordonii*
11 *1A*	No Identification	*Peptostreptococcus* sp.
12 *2A*	*A. naeslundii*	*S. intermedius*
15 *1A*	No Identification	*Bacillus* sp. possibly *B. cereus*

KEY:

A. , *Actinomyces*; *E.*, *Eubacterium*; *P.*, *Prevotella*; *S.*, *Streptococcus*

FOOTNOTES TO TABLE 3.15.

1. Those isolates listed as No Identification, produced either 'questionable code' with RapID ANA II system or 'unacceptable profile' with Rapid ID 32 A (see table 3.11).
2. With the exception of isolate **5** *1A*, all commercial identifications listed were above 79.9%.
3. The reason for the choice of commercial identification kit is discussed in sections 2.10.1.
4. The reader is referred to tables 3.11 for identification kit results, 3.12 for the Gram morphologies of these isolates, 3.13 for results of other tests and 3.14 for GLC results.
5. An explanation for the reasons that these isolates were subjected to sequence analysis rather than having the identifications accepted is provided in section.

6. The suggested identifications from sequence analysis were compared with all previous test results and they are discussed in relation to these and with reference to the final identification in section 3.13.4.
7. *Pseudomonas* sp. was the result of contamination during the sequencing process, for further details see section 4.11.11.
8. A discussion of 16S rRNA gene sequencing in relation to bacterial identification can be found in section 4.5.5 and 4.5.6.

FINAL IDENTIFCATIONS OF ANAEROBIC AND FACULATATIVE ISOLATES FROM PLS PATIENTS (TABLES 3.16 AND 3.17)

Table 3.16 Obligately anaerobic species isolated from the oral cavity of PLS patients

CLINICAL ISOLATE REFERENCE	IDENTIFICATION
1 *2A*	*Eubacterium* sp.
1 *2B*	*P. nigrescens*
3 *1D*	*Pstr. micros*
3 *2A*	*P. nigrescens*
5 *1D*	*P. intermedia*
5 *2B*	*P. melaninogenica*
6 *2A*	*P. melaninogenica*
8 *1A*	*Prevotella* sp. possibly *P. oralis*
11 *1A*	*Peptostreptococcus* sp.
17 *2A*	*P. melaninogenica*
18 *2A*	*Pstr. anaerobius*

Table 3.17 Facultative species isolated from the oral cavity of PLS patients

CLINICAL ISOLATE REFERENCE	FINAL IDENTIFICATION
1 *1B*	*A. israelii*
2 *1B*	*Mobiluncus* sp.
2 *2A*	*Leuconostoc* sp.
2 *2B*	*Streptococcus* sp.
3 *1A*	*Actinomyces* sp.
6 *1A*	*S. sanguis*
7 *2B*	*S. gordonii*
12 *2A*	*S. intermedius*
15 *1A*	*B. cereus*
18 *1D*	*Gemella morbillorum*

Table 3.18 Identification of isolates which was not accepted

These isolates therefore remain unidentified.

CLINICAL ISOLATE REFERENCE	IDENTIFICATION
5 *1A*	*Pseudomonas* sp.

KEY:

A. , *Actinomyces*; *B.*, *Bacillus*; *P.*, *Prevotella*; *Pstr.*, *Peptostreptococcus*; *S.*, *Streptococcus*

Using the test results in table 3.13 and Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993) these identifications were checked. The profiles fit that of *Bacteroides* species, the previous taxonomic status of *Prevotella* species and GLC analysis matched this identification, with peaks of acetic, *iso*-butyric and *iso*-valeric acids.

One isolate (**18** *1D*) was identified as *Gemella morbillorum*, this Gram-positive coccus demonstrated negative catalase and oxidase reactions and an ability to grow under aerobic conditions. The only major end-product of metabolism was lactic acid which agreed with this species identification.

3.13.4.1.2 Identification by RapID ANA II alone

Three isolates (**2** *1B*, **3** *1D* and **18** *2A*) were identified by RapID ANA II system in addition to the confirmation of identification of the three isolates identified as *P. intermedia* (**1** *2B*, **3** *2A* and **5** *1D*). Two Gram-positive coccoid isolates (**3** *1D* and **18** *2A*) were identified as *Peptostreptococcus micros* and *P. anaerobius* respectively with identity levels of 98%. Both isolates produced small amounts of volatile acid end-products but displayed no major peak by GLC analysis, however the identifications were still accepted as neither isolate was shown to grow under aerobic conditions and both demonstrated negative catalase and oxidase tests.

Isolate **2** *1B* was identified as *Mobiluncus* species, an identification that was accepted despite the lack of an acetic acid peak in the GLC traces and a Gram-positive Gram stain result.

3.13.4.1.3 Identification by Rapid ID 32 Strep alone

A single Gram-positive isolate, **2** *2A* was identified by this kit. An identification of *Leuconostoc* species was accepted with an identity level of 75.6%. The second choice identification was *Lactococcus lactis cremoris* with 24% probability of correct identification. This genus identification agreed with the negative catalase and oxidase reactions and the ability of this isolate to grow in an aerobic environment. GLC profiles were as expected for *Streptococcus*, *Gemella* or *Leuconostoc*.

3.13.4.2 Identification of those isolates with high identity levels by commercial identification kit which was questioned

Six isolates (**4** *1E*, **4** *2A*, **10** *1B*, **10** *2A*, **10** *2B* and **12** *2A*) were identified to high identity levels (80%) with commercial identification kits but were subsequently discounted. Two isolates were identified as *Clostridium* species. Isolate **4** *2A* was identified as *Clostridium sporogenes*, an identification that was discounted due to the positive catalase test, negative spore stain and a GLC profile suggestive of *Eubacterium* spp. which are catalase negative. Unfortunately this isolates could not be tested further. Isolate **10** *2A* was identified as *Clostridium sordelli* with an identity level of 92.4%. The Gram stain revealed Gram-negative rods with the characteristic shape of *Fusobacterium nucleatum*, therefore the identification of *C. sordelli* was rejected in favour of the second choice *F. nucleatum* with an identity level of only 3.8%. Although the Gram stain was conclusive, the lack of further evidence due to loss of viability and the low identity level meant this identification could not be accepted.

Isolate **4** *1E* was identified as *Actinomyces meyeri* with an identity level of 95.3%, however, this identification was discounted due to the Gram-negative staining of this isolate. The isolate died, therefore a young culture was not available to be Gram stained or for GLC analysis to check the validity of this result.

Two other isolates (*10* **1B** and *12* **2A**) were tentatively identified as *Actinomyces* species. Despite the corresponding Gram stain of isolate **10** *1B* (Gram-positive bacilli), the identification of *A. israelii* obtained using the RapID ANA II system was questioned due to the positive catalase test of this isolate, pink colony colour (*A. israelii* is white) and GLC analysis which yielded no major end-products of metabolism, suggesting *Eubacterium* species. No conclusive identification was reached before this isolate died. The identification of isolate **12** *2A* as *A. naeslundii* was also discounted due to the positive catalase test and the coccoid cells seen by Gram stain. The production of a major lactic acid peak by GLC analysis and the Gram morphology matched the identification of *S. intermedius* obtained by 16S rRNA gene sequencing.

The identification of isolate **10** *2B* as *Peptostreptococcus* sp. was discounted due to the rod shaped cells. GLC analysis suggested *Eubacterium* which correlated with the Gram stain morphology but the facultative nature of this isolate discounted this identification. This isolate could not be assigned to a genus on the basis of other tests and no conclusive identification was reached.

3.13.4.3 Identification of those isolates with low identity levels by commercial identification kit which was not accepted

Fourteen (**1** *1B*, **1** *2A*, **2** *2B*, **3** *1A*, **3** *2B*, **5** *1A*, **5** *1B*, **6** *1A* , 7 *2B*, **10** *1A*, **11** *1A*, **13** *2A*, **14** *2E* and **15** *1A*) isolates were unidentified by commercial identification kits. Of these, 7 (**1** *1B*, **1** *2A*, **3** *1A*, **5** *1A*, **5** *1B*, **13** *2A* and **14** *2E*) had an identification value below 79.9% which could not be considered significant. The Gram-positive bacilli, **5** *1A* and **5** *1B* were tested using RapID ANA II system and Rapid ID 32 A respectively. The suggested taxa for both isolates included *Bifidobacterium* and *Actinomyces*. Either identification could be correct, for isolate **5** *1A* on the basis of the Gram morphology, negative catalase and oxidase reactions, lack of growth under aerobic conditions and GLC profile, and for isolate **5** *1B*, the Gram stain result. No conclusion was reached for **5** *1B*, due to the loss of viability of the isolate. The GLC profile of isolate **5** *1A* suggested *Actinomyces*, the isolate was available for partial 16S rRNA sequencing analysis but an identification of *Pseudomonas* species was achieved by this method presumed to be a result of contamination during the sequencing procedure.

No significant identification of isolate **1** *2A* was made using Rapid ID 32 A. GLC analysis yielded only small amounts of lactic acid and the absence of any major products suggests *Eubacterium*. Negative catalase and oxidase tests and the obligate anaerobic nature of this isolate confirmed this identification and a genus level identification was accepted.

A low level identification of *Peptostreptococcus anaerobius* was achieved for isolate **13** *2A* which were not enhanced by the Gram morphology, GLC or other tests namely the positive catalase test and the ability to grow in an aerobic environment. No genus level identification could be assigned to this isolate.

An identification level of 79.7% *Eubacterium aerofaciens* was obtained for isolate **1** *1B*, however, the occurrence of major lactic and succinic acid peaks seen with GLC analysis suggest an identification within the genus *Actinomyces*. For this catalase and oxidase negative, Gram-positive bacilli, either identifications would be satisfactory but ultimately an identification of *A. israelii* was achieved when sequence analysis was undertaken. Another isolate which appeared to fall into two genera was **3** *1A*, the RapID ANA II system suggested either *Bifidobacterium* or *Actinomyces*. The major lactic and succinic acid peaks seen by GLC analysis favoured *Actinomyces* and sequence analysis confirmed this with an identification of *Actinomyces* sp.

A Gram-negative bacillus (**14** *2E*) was incorrectly determined to be *Clostridium difficile*. In addition to the inconsistent Gram morphology, negative catalase tests and spore stain and positive oxidase test discounted this identification. This information and the inability of this isolate to grow under aerobic conditions suggested *Eikenella* but this could not be confirmed.

3.13.4.4 Identification of those isolates with no identification by commercial identification kit

Of the six further isolates (**2** *2B*, **6** *1A*, 7 *2B*, **10** *1A*, **11** *1A* and **15** *1A*) which had no acceptable profile with identification kits, genus level identifications could be suggested in 4 cases, on the basis of other work. Isolate **15** *1A* also displayed catalase activity, but the ability to grow under aerobic conditions suggested *Listeria* or *Bacillus*. An identification of *Bacillus cereus* was confirmed by partial 16S rRNA gene sequencing, despite the ability of this isolate to grow under anaerobic conditions. Isolate **6** *1A*, gave no acceptable profile when tested with RapID ANA II, but the Gram morphology and the results of other tests including GLC, suggested *Gemella*, *Leuconostoc* or *Streptococcus*. An identification of *S. sanguis* was obtained by partial 16S rRNA gene sequencing. This could have been verified by use of the Rapid ID 32 Strep kit. Isolates **2** *2B* and **11** *1A* recorded unacceptable profiles with the RapID ANA II system. Further tests and Gram morphology suggest an identification of *Streptococcus* or *Peptostreptococcus*. The lack of growth under aerobic conditions of isolate **11** *1A* favours *Peptostreptococcus* whilst **2** *2B* may belong to the streptococci. GLC analysis of isolate **11** *1A* yielded a complex profile similar to that obtained for *Peptostreptococcus anaerobius* (Holdeman *et al.*, 1977) but suggesting the species. Partial 16S rRNA gene sequencing confirmed this identification. GLC analysis of isolate **2** *2B* gave a trace indicating *Streptococcus*, *Gemella* or *Leuconostoc*, therefore a genus level identification of *Streptococcus* was accepted.

The Gram positive bacilli **10** *1A* had a GLC profile fitting with an identification of *Eubacterium*, with a major succinic acid peak and a minor fumaric acid peak was seen, however the positive catalase test rules out this identification. On the basis of the catalase and oxidase reactions and the lack of growth in an aerobic environment, this isolate fits the genus *Propionibacterium*, however, no conclusive identification was reached.

Isolate 7 *2B* demonstrated characteristics; positive catalase test and negative oxidase test, typical of and a Gram morphology fitting the genus *Propionibacterium*. GLC analysis

suggested *Actinomyces* which does not fit with the other characteristics. An identification of *S. gordonii* was made by 16S rRNA gene sequencing. Streptoccci have been reported to form rod-shaped cells (Tanner *et al.*, 1994) and if the correct Gram morphology of streptococci was used to analyse GLC profiles, the results would enhance this identification. Due to the high level of identity with *S. gordonii* this identification was accepted.

3.13.4.5 Unidentified isolates

A total of 15 isolates were unidentified, 14 (**3** *1B*, **3** *1E*, **3** *2B*, **4** *2A*, **4** *1E*, **4** *2B*, **5** *1B*, **7** *1A*, **7** *1B*, **10** *1B*, **10** *2A*, **10** *2B*, **11** *2A* and **13** *2A*) due to loss of viability and an additional one (**5** *1A*) due to contamination during the 16S rRNA sequencing procedure.

No identification was reached for isolates **10** *1A* and **14** *2E* for reasons described previously.

Table 3.19 Summary of the total number of clinical isolates recovered, lost and identified

Total number of clinical isolates	91
Number of clinical isolates lost	26
Number of isolates unidentified	6
Number of isolates identified to at least genus level	59
Number of isolates identified as *P. intermedia* / *P. nigrescens*	3

3.14 DETERMINING THE PRESENCE OR ABSENCE OF SPIROCHAETES IN GINGIVAL SAMPLES

No attempt was made to speciate or quantify spirochaetes seen within the gingival plaque of PLS patients but the presence of numerous spirochaetes was recorded in plaque samples taken from both PLS patients.

3.15 FURTHER IDENTIFICATION OF ANAEROBES IDENTIFIED AS *P. INTERMEDIA* BY RAPID ID 32 A

As described in section 3.13.4.1.1 three clinical isolates (**1** *2B* referred to as A, **3** *2A* referred to as D and **5** *1D* referred to as C) from 2 PLS patients were identified as *P. intermedia* using both the Rapid ID 32 A kit and the RapID ANA II system. The identities of two isolates were confirmed as *P. nigrescens* and the third as *P. intermedia* by partial 16S rRNA gene sequencing (see section 3.13.3.3 and table 3.15).

A fourth isolate (**2** *3A* referred to as B) also identified as *P. intermedia* by Rapid ID 32 A and RapID ANA II was also tested further.

3.15.1 COMMERCIAL IDENTIFICATION KITS RAPID ANA II SYSTEM AND RAPID ID 32 A

Both the Rapid ID 32 A kit and the RapID ANA II system were evaluated for their ability to identify *P. intermedia* and *P. nigrescens*. In all cases an identification of *P. intermedia* was made. The individual tests results can be seen in table 6.22 (appendix 6.22). It is interesting to note that the only differences between the four isolates is the absence of arginine hydrolysis by **1** *2B* (*P. nigrescens*) when tested with RapID ANA II. There are more discrepancies between results with Rapid ID 32 A. Both isolates identified as *P. nigrescens* (**1** *2B*, **3** *2A*) were positive for mannose (MNE) and raffinose (RAF) fermentation, whilst neither *P. intermedia* isolate was positive for MNE, **2** *3A* was positive for RAF. Isolate **1** *2B* failed to react to PAL (phosphatase alcaline) or FUC (α-fucosidase) where the other three isolates did.

Test results between the two kits were not always comparable. In all cases the four isolates are positive for alpha glucosidase (αGLU), indole production (IND) and leucyl-glycine arylamidase action (LGA; Rapid ID 32A, LGY; RapID ANA II). However, in the case of arginine hydrolysis (ArgA; Rapid ID 32A, ARG, RapID ANA II) by arginine arylamidase, isolate **1** *2B* tested negative with RapID ANA II and isolate **5** *1D* tested negative with Rapid ID 32 A. Similarly all isolates were positive for alpha fucosidase activity under the RapID ANA II system (αFUC) but **1** *2B* failed to react when tested in the Rapid ID 32 A kit (FUC). The Rapid ID 32 A kit contains more tests which cannot be compared.

3.15.2 TRADITIONAL IDENTIFICATION TESTS

All 4 isolates were small Gram-negative rods which were catalase and oxidase negative, obligately anaerobic and displayed black-pigmenting colonies when grown on blood-FAA. When grown on egg yolk agar some lipase activity was detected after 3 days.

3.15.3 GLC

3.15.3.1 Volatile acids and alcohols

The GLC profile of all 4 isolates was identical, each displayed large amounts of acetic acid, *iso*-butyric and *iso*-valeric acids. A typical gas chromatogram is illustrated in figure 3.19 for isolate **3** *2A*. Results obtained by GLC analysis confirm the identity of these 4 isolates within the genus *Prevotella* (table 3.14), but more specifically as *P. intermedia* (Summanen *et al.*, 1993). There was no differences in the traces obtained between *P. intermedia* and *P. nigrescens*.

3.15.4 RAPD-PCR

The four clinical isolates were amplified by primer L10 as described in section 2.5.8. PCR products were separated by electrophoresis in parallel with known strains of *P. intermedia* and *P. nigrescens*. This is shown in figure 3.11 and described in section 3.7. By this technique, isolates B and C are *P. intermedia* (amplifying specific bands II and IV) and isolates A and D share bands (I and III) with *P. nigrescens*.

3.15.5 PARTIAL SEQUENCING OF 16S RRNA GENE

Isolates A-D were sequenced and the results analysed as described in section 2.6 and illustrated in appendix 6.8.

Isolates A and D showed greatest similarity to *P. nigrescens* and isolates B and C to *P. intermedia*.

Table 3.20 summarises the identification achieved for each isolate using these techniques.

3.15.6 PCR WITH *P. INTERMEDIA* AND *P. NIGRESCENS* SPECIFIC PRIMERS

As described previously, a *P. intermedia*-specific (P-int) and a *P. nigrescens*-specific (P-nig) primer had been designed. These primers were tested for their ability to amplify DNA from the four clinical isolates. In all cases amplification was specific, agreeing with the identifications obtained by sequence analysis (see section 3.13.3.3).

Table 3.19 Summary of the identifications of 4 clinical isolates

TECHNIQUE	**1** *2B* A	**2** *3A* B	**3** *2A* C	**5** *1D* D
Traditional tests	*Prevotella*	*Prevotella*	*Prevotella*	*Prevotella*
GLC	*P. intermedia*	*P. intermedia*	*P. intermedia*	*P. intermedia*
RapID ANA II	*P. intermedia*	*P. intermedia*	*P. intermedia*	*P. intermedia*
Rapid ID 32 A	*P. intermedia*	*P. intermedia*	*P. intermedia*	*P. intermedia*
RAPD-PCR	*P. nigrescens*	*P. intermedia*	*P. nigrescens*	*P. intermedia*
PCR	*P. nigrescens*	*P. intermedia*	*P. nigrescens*	*P. intermedia*
16S rRNA gene	*P. nigrescens*	*P. intermedia*	*P. nigrescens*	*P. intermedia*
Final Identification	*P. nigrescens*	*P. intermedia*	*P. nigrescens*	*P. intermedia*

3.16 FURTHER EXAMINATION OF PINLOs

Three PINLOs; A391, HST 1156 and HST 2160 were available during this study.

3.16.1 COMMERCIAL IDENTIFICATION KITS RAPID ANA II SYSTEM AND RAPID ID 32 A

All 3 PINLOs were tested by both the RapID ANA II system and the Rapid ID 32 A kit and in all cases an identification of *P. intermedia* was achieved. The test results are given in appendix 6.23, tables 6.23 and 6.24 in comparison with P. *corporis*, *P. pallens* and the 4 clinical isolates. When tested by RapID ANA II system, all 3 strains gave identical profiles which differed from the clinical *P. intermedia* and *P. nigrescens* only in the negative αFUC test. Therefore, the same 6-digit microcode was obtained for all 3 strains by the RapID ANA II system which gave a identification of *P. intermedia* (99.9% satisfactory).

As with the clinical *P. intermedia* and *P. nigrescens* isolates, using the Rapid ID 32 A kit demonstrated more reactivity differences between the strains. Strains HST 1156 and HST 2160 were positive for MNE and RAF were A391 was not. A391 differed by displaying histidine arylamidase (HisA) activity. The negative PAL test (phosphatase alcaline) was the consistent feature which differed between the test results of the PINLOs and isolates **2** *3A*, **3** *2A* and **5** *1D*. The PINLOs also demonstrated glutamyl glutamic acid arylamidase activity (GGA) which was seen only when **1** *2B* and **2** *3A* were tested. The profile of **1** *2B* directly matched that of PINLOs HST 1156 and HST 2160. The 10-digit microcode generated by the Rapid ID 32 A kit differed by three digits between the profile of A391 and that of the other 2 strains, but the identification for both was *P. intermedia* (99.9% excellent identification).

The PINLOs were positive for arginine hydrolysis (ArgA; Rapid ID 32A, ARG, RapID ANA II), alpha glucosidase (αGLU), indole production (IND) and leucyl-glycine arylamidase action (LGA; Rapid ID 32A, LGY; RapID ANA II) by both kits.

A FASTA search and distance analysis with PINLO partial sequences revealed a closest phylogenetic relationship with *P. corporis* (section 3.9.2). The introduction (section 1.11) describes how some weakly pigmenting isolates described as PINLOs were reclassified as *P. pallens*. For this reason, representatives of these strains were also examined by commercial identification kit (tables 6.23 and 6.24). Using Rapid ID 32 A, three strains of *P. corporis* and two of *P. pallens* were identified as *P. intermedia*, and although all the profiles were different from those previously tested, like the PINLOs and the clinical isolates, *P. pallens*

and *P. corporis* demonstrated positive ArgA and LGA tests. Strains of *P. pallens* were identical to each other but for the positive αFUC reaction of *P. pallens* ANH 9423, and the positive SER reaction of the type strain *P. pallens* NCTC 130Y2 which yielded a profile identical to the PINLOs. The microcodes were unique to each strain and identifications of *P. intermedia* (99.99% implicit and 99.98% adequate respectively) were obtained. Strains of *P. corporis* gave similar profiles to the PINLOs, the only difference being a negative indole reaction. Both *P. corporis* and *P. pallens* were identified as *P. intermedia.*

With the RapID ANA II system, *P. corporis* strains differed from PINLOs due to the negative indole reaction whilst *P. pallens* NCTC 130Y2 differed only by a positive serine hydrolysis (SER) test and *P. pallens* AHN 9423 by the positive FUC reaction. The microcode was identical for all three strains, giving an implicit identification of *P. corporis.*

The discrepancies between the test results obtained for *P. corporis* and *P. pallens* occurred in the serine hydrolysis reaction (SER; RapID ANA II, SerA; Rapid ID 32 A) of *P. pallens* NCTC 130Y2, the RapID ANA II system reported a positive reaction not seen using Rapid ID 32 A, the positive αFUC reaction of *P. pallens* AHN 9423 and the negative indole test of *P. corporis* strains. *P. pallens* strains were identified as *P. intermedia.*

3.16.2 TRADITIONAL LABORATORY IDENTIFICATION TESTS

All 3 isolates were small Gram-negative rods which were catalase and oxidase negative, obligately anaerobic and displayed black-pigmenting colonies when grown on blood-FAA. When grown on egg yolk agar some lipase activity was detected after 3 days.

3.16.3 GLC

GLC analysis produced profiles similar to those for *P. intermedia.* A typical gas chromatogram produced by the analysis of volatile acids and alcohols is illustrated in figure 3.19 for *Prevotella* spp.

3.16.4 RAPD-PCR

As described in section 3.8, 3 PINLOs were examined by RAPD-PCR and profiles were compared with other *Prevotella* species. The banding patterns were different from the other *Prevotella* species tested and cluster analysis confirmed this identity (see section 3.8).

3.16.5 PARTIAL SEQUENCING OF 16S RRNA GENE

The 3 PINLO strains were sequenced and analysed as described previously. A FASTA search and cluster analysis of sequence distance data suggested a closest phylogenetic relationship with *P. corporis*.

3.16.6 PCR WITH *P. INTERMEDIA* AND *P. NIGRESCENS* SPECIFIC PRIMERS

As described in sections 3.11.3, primers P-int and P-nig were tested for cross reactivity with a range of organisms including PINLOs. No cross reactivity was seen with P-int, however one strain HST 1156 was amplified by P-nig.

4. DISCUSSION

This thesis contains work which has been split into two main topic areas, the first DNA based techniques (PCR, RAPD-PCR and partial 16S rRNA gene sequencing) used for the differentiation, identification and characterisation of members of the genus *Prevotella*. The second, clinical microbiology encompassing the identification of clinical bacterial isolates from PLS patients, *P. intermedia* and *P. nigrescens* isolates and PINLOs by commercial and traditional means. These two aspects of the work are drawn together by the continued examination of *P. intermedia* and *P. nigrescens* isolated from PLS patients by the DNA based techniques of RAPD-PCR and partial 16S rRNA gene sequencing.

4.1 *PREVOTELLA INTERMEDIA* AND *P. NIGRESCENS*

Prevotella intermedia and *P. nigrescens* are black-pigmented bacteria with an oral habitat. They can be successfully maintained in the laboratory under anaerobic conditions, on solid medium supplemented with blood and in other liquid medium. The 12 test strains (listed in table 2.1) were available at the start of the study. All microorganisms used during this study were preserved in FUM medium (Gmür and Guggenheim, 1983) originally described by Loesche *et al.* (1972) and used previously for the cultivation of *P. intermedia*, *P. nigrescens*, (Devine *et al.*, 1994) and other *Bacteroides* (Gmür and Guggenheim, 1983). Its components include haemin which is required for cytochrome production, menadione (vitamin K) which is required for menaquinone biosynthesis and cysteine which is a growth requirement and a reducing agent (Shah, 1992).

The association of *P. intermedia* and *P. nigrescens* in oral health and disease is discussed in the introduction section 1.9.3 and the differing site specificities and roles are accepted (Devine *et al.*, 1994). The phenotypic similarities (introduction section 1.9.2) of the two species and the inability to distinguish them by use of routine laboratory tests (introduction sections 1.10.1, 1.10.2, 1.10.3, 1.10.5) has lead to many techniques being tried (introduction sections 10.1.4, 1.10.6-1.10.14). Techniques which will determine the distribution of the species within the oral cavity, study the clonal analysis of isolates and clarify the associations of each species with disease status and possible aetiological role (Mättö *et al.*, 1996b) are desirable. As new molecular techniques are used, information is provided which may influence the current taxonomic status of species and so alter our knowledge of the bacterial kingdom.

DISCUSSION

SECTION A: MOLECULAR (DNA) TECHNIQUES

4.2 DNA EXTRACTION

DNA was extracted from Gram-negative organisms for RAPD-PCR analysis and PCR and from all organisms, both Gram-positive and Gram-negative, which were to be analysed by partial 16S rRNA gene sequencing. The use of broth cultures for DNA extraction is commonly reported (Brikun *et al.*, 1994; Farber and Addison, 1994; Österlund and Engstrand, 1995 and Welsh *et al.*, 1992), although the use of plate cultures is not unusual (MacGowan *et al.*, 1993; Smith *et al.*, 1989a, b). The use of pure cultures for DNA extraction is very important, Gram stains were checked against culture history before the extraction procedure was carried out and any questionable results were discarded. Purity plates were prepared at the same time and examined closely for contaminants, extractions from any contaminated purity plates were discarded.

During the DNA extraction procedure, multiple cultures were tested, therefore the importance of not mixing up eppendorfs or haphazard labelling should be remembered. If at any time a sample was found unlabeled or the label accidentally removed, the extraction was not continued for that sample. The use of phenol, chloroform and ethanol during the extraction procedure are steps during which ink can be removed accidentally.

The methods were different for Gram-positive and Gram-negative organisms and both are discussed below.

4.2.1 GRAM-NEGATIVE ORGANISMS

All DNA extraction from Gram-negative organisms was performed using a phenol-chloroform extraction adapted by Dr. M Anderson (Biological Sciences, University of Manchester).

Cells are resuspended in an extraction buffer, STE, which in this study contained sucrose, Tris-chloride and EDTA (section 2.2 for components, 2.4.1 for use). EDTA chelates magnesium ions, which if not removed enhance nucleic acid and protein associations, inhibits nucleases and prevents the formation of magnesium hydroxide which will lower the pH. A pH of 8.0 is required (and given by the presence of Tris-Cl at pH 8.0) to remove DNA to the aqueous phase and it will also partition RNA (Wallace, 1987a). A high salt concentration is beneficial in aiding partition of nucleic acid to the aqueous phase (Wallace, 1987a).

Lysosyme is used to damage the integrity of the bacterial cell wall (as described in the introduction section 1.2.3.3) to release the DNA. Removal of Mg^{2+} by EDTA as described above, weakens the ionic crosslinks of the lipids of the outer membrane (Gram-negative

bacteria) allowing the lysosyme to permeate and attack the peptidoglycan. A hypertonic solution (having higher osmotic pressure) will cause water to leave the bacterial cell and separate the cell membrane from the cell wall which accounts for the presence of sucrose in the extraction buffer. As a consequence of this action, spheroplasts are formed.

The spheroplasts are lysed by detergent which lowers the surface tension disrupting lipids in the cell membrane. The ionic detergent sodium-dodecyl-sulphate (SDS) also helps dissociation of proteins and also to inhibit endogenous nucleases which are released (Wallace, 1987a), addition of proteinase K at this point also aids protein dissociation and reduces the total amount of protein (Wallace, 1987a). A high temperature prevents precipitation of SDS (Wallace, 1987a).

The addition of phenol and chloroform and subsequent centrifugation result in the protein being within the lower organic phase and the white middle layer and the nucleic acid in the upper phase. Chloroform is used because of its high density and therefore efficient separation of the aqueous and organic phases and also because it is able to denature protein affecting its dissociation from the nucleic acid (Wallace, 1987a). Isoamyl alcohol is added to chloroform to prevent foaming (Wallace, 1987a).

The aqueous phase is removed and contains the nucleic acid which is extracted by ethanol precipitation. In this study, precipitation with isopropanol was performed first and followed by an ethanol precipitation, an immediate ethanol precipitation is necessary as salts from the original sample precipitate with the DNA (Wallace, 1987). At a low temperature, the isopropanol (ethanol), nucleic acid and salt that is added (NaCl) will precipitate (Wallace, 1987b). NaCl also helps remove any excess SDS by keeping it in solution (Wallace, 1987a). The addition of ammonium acetate as the next step is to remove salts and protein and prevent precipitation of the nucleic acid (Wallace, 1987b). Ethanol precipitation with 100% ethanol is followed by 70% ethanol (Wallace, 1987b) which removes organic and inorganic contaminants (Bickley and Owen, 1995). Extracted DNA is stored in TE buffer (for components see section 2.2) at 4°C, it contains EDTA essential to chelate magnesium ions required by DNases and therefore prevent their activity (Wallace, 1987b).

As mentioned above, the use of a pH above 8.0 results in the extraction of DNA and RNA, a lower pH results in only RNA in the aqueous phase (Wallace, 1987a). If the presence of RNA is undesirable, it is readily removed by the addition of RNase (Smith *et al.*, 1989b).

This method was accepted without alteration due to the success of the protocol for DNA extraction from oral treponema.

4.2.2 GRAM-POSITIVE ORGANISMS

DNA was extracted from Gram-positive organisms using a commercially available kit rather than the procedure described above. This was for two reasons. Firstly, the peptidoglycan layer of Gram-positive organisms, although not protected by an outer membrane, consists of many layers of peptidoglycan with extensive cross-links. Unlike the peptidoglycan of Gram-negative organisms, that of Gram-positive organisms is not very permeable. This makes lysosyme less effective against Gram-positive bacteria. To lyse the cell wall, additional enzymes such as mutanolysin (Bickley and Owen, 1995; Parrish and Greenberg, 1995) or lysostaphin (Bickley and Owen, 1995; Stackebrandt and Liesack, 1993) would be required or the cells' own autolytic enzymes could be exploited with a hypertonic medium. The second reason for turning to a commercial kit was that initially extractions from Gram-positive cultures were anticipated only for limited numbers of samples; therefore for speed, cost effectiveness and ease it was decided to try a commercial kit rather than experiment with altering the protocol.

The composition of solutions was not provided but the components can be speculated on, based on an understanding of the extraction procedure.

Use of the kit began with protoplast formation (equivalent to spheroplasts of Gram-negative organisms) in cell suspension solution and lytic enzyme solution probably containing salts, sucrose and EDTA as well as at least one lytic enzyme. Cell lysis solution followed which probably contained SDS and either pronase K (Wallace, 1987a) or proteinase K and the protein precipitation solution probably contained ammonium acetate. Isopropanol and ethanol is used to precipitate the DNA, which is resuspended in DNA hydration solution which would have included EDTA. No phenol-chloroform is used during this procedure, and RNase is used decreasing the influence of pH. In addition only one step is used to separate protein and nucleic acid and precipitate residual SDS.

A Puregene DNA Isolation Kit is available for Gram-negative organisms and the anticipated yield is higher than that for Gram-positive organisms (Puregene DNA Isolation Kit Instruction booklet).

4.2.3 TRADITIONAL EXTRACTION PROCEDURES COMPARED TO COMMERCIAL EXTRACTION KITS

Traditional phenol-chloroform methods have the disadvantage of being time consuming.

The Puregene DNA isolation kit required only 1 ml of culture from which DNA was extracted compared to the 5 ml used for phenol-chloroform. Gram-negative cells were

resuspended in 1 ml of cell extraction buffer, however were likely to obtain a higher concentration of cells than 1 ml of Gram-positive culture therefore improving the chances of obtaining a good yield of DNA. To increase the yield of DNA from Gram-positive cultures, 1ml was removed only when the culture was very turbid.

The Puregene DNA isolation kit could be adapted for 5ml cultures, but owing to the cost of the kit would not have been sensible. Only 30-90 ng per sample of purified PCR product was required for partial 16S rRNA gene sequence analysis of Gram-positive bacteria, therefore it was not necessary to scale up the protocol.

The Puregene DNA isolation kit was very quick to use, taking only 1-2 h, whilst the Gram-negative protocol was spread over two working days. Although it is convenient to perform quick experiments, the length of the extraction protocol did not cause significant problems in this case. However, the extraction of pure DNA using either a commercially available kit or traditional procedure is preceded by the necessity of several days cultivation of bacteria before extraction.

Greater concentrations of DNA was routinely extracted from Gram-negative bacteria, however for this study DNA from Gram-negative bacteria was used in larger amounts, not only because it was used for several techniques but because it was needed repeatedly for RAPD-PCR. If large amounts of DNA are required on a regular basis, it is suggested that traditional procedures are adopted as used routinely commercial extraction kits would prove to be expensive and with the Puregene DNA isolation kit, the protocol would have to be scaled up.

DNA extracted by both techniques should have been free from protein, although this was not tested. DNA extracted using a commercial extraction kit was free from RNA, however, RNA has been shown to have no affect on RAPD-PCR (Ménard *et al.*, 1992). Ultra-pure template is not required for conventional PCR (Williams, 1989) and owing to the fact that amplified product is seen from template extracted in different ways, (Agersborg *et al.*, 1997; section 4.6.8), RNA does not adversely affect the PCR.

4.2.4 ALTERNATIVE DNA EXTRACTION PROCEDURE

Many methods have been described to obtain DNA suitable for use for PCR based techniques, the implications of alternative techniques for RAPD-PCR results is discussed in section 4.4.11). Crude cell extracts can be obtained by boiling colonies from plates or cells from liquid broth (Brikun *et al.*, 1994; Welsh and McClelland, 1990) in sterile distilled water (Linton *et al.*, 1994) or Tris (pH 7.0; Corney *et al.*, 1993), suspending in sterile

distilled water and shaking with glass beads (Johansson *et al.*, 1995), cycles of boiling in sterile distilled water then freezing in ethanol and dry ice (Johansson *et al.*, 1995) or incubation of cells with buffer and proteinase K (Johansson *et al.*, 1995) or detergent and proteinase K (Linton *et al.*, 1994). All methods are followed by briefly centrifuging the sample to pellet cell debris (Johansson *et al.*, 1995; Linton *et al.*, 1994) and have been tested for both Gram-negative (Brikun *et al.*, 1994) and Gram-positive (Johansson *et al.*, 1995; Linton *et al.*, 1994) bacteria. Many commercial extraction kits are available and have been compared by other researchers (Linton *et al.*, 1994).

The method used in this study to extract DNA from Gram-negative organisms is similar to those reported elsewhere. Changes in protocol involve constituents of buffers (Brikun *et al.*, 1994) or the use of additional chloroform extractions either to remove RNase (Corney *et al.*, 1993; Ménard *et al.*, 1992), or prior to a phenol:chloroform extraction to remove lipids (Wallace, 1987a) and cellular polysaccharides (Smith *et al.*, 1989b) or after a phenol:chloroform extraction to remove trace phenol (Myers *et al.*, 1993). Hexadecyltrimethyl ammonium bromide (CTAB) is often used (Ménard *et al.*, 1992; Smith *et al.*, 1989a, b) with SDS and proteinase K to promote the removal of proteins, polysaccharides and other cellular debris (Smith *et al.*, 1989b). Guanidium isothiocyanate is often used in extraction protocols to denature proteins, disrupt cell walls and inactivate nucleases (Bickley and Owen, 1995). Parrish and Greenberg (1995) reported an extraction protocol which utilised lysosyme, mutanolysin and guanidium isothiocyanate to lyse bacterial cells and was successful for both Gram-negative and Gram-positive organisms as well as the mixed population of dental plaque to allow PCR analysis. The procedure followed in this study would not be suitable for the extraction of mixed flora DNA without modification due to the occurrence of Gram-positive organisms, the Puregene DNA isolation kit could be used for this purpose.

4.3 DNA QUANTIFICATION

Extracted DNA was quantified during this study by agarose gel electrophoresis, a method recommended for small amounts of nucleic acid or impure extractions (Sambrook *et al.*, 1987) and a technique commonly used for RAPD-PCR studies (Aufauvre-Brown *et al.*, 1992; Brikun *et al.*, 1994; Corney *et al.*, 1993; Ménard and Mouton, 1993; Ménard *et al.*, 1992; Welsh and McClelland, 1990). The importance of the amount of DNA required for RAPD-PCR is questioned (see section 4.4.7) and the lack of any attempt to quantify DNA for use in RAPD-PCR has been reported (MacGowan *et al.*, 1993).

As described in section 1.8.5.5 ethidium bromide intercalates into the DNA and fluoresces, the amount of fluorescence being proportional to the total amount of DNA thus enabling quantification against known standards (Sambrook *et al.*, 1987). The measurement of brightness is subjective and probably varies amongst individuals. This technique should detect as little as 1-5 ng of DNA (Sambrook *et al.*, 1987), however in this study standards below 10 ng were not routinely visible. Alternative quantification procedures are described (Sambrook *et al.*, 1987). Spectrophotometry is often used (Ménard *et al.*, 1992) and is suitable when there are large amounts of pure DNA but phenol from an extraction procedure or large amounts of protein will affect the accuracy of this technique (Sambrook *et al.*, 1987). Optical density readings are taken at wavelengths of 260 nm and 280 nm and the ratio OD_{260}/OD_{280} provides the estimated purity since pure DNA has a value of 1.8 (Sambrook *et al.*, 1987). The OD is affected by whether the DNA is double or single stranded (Sambrook *et al.*, 1987). Sambrook *et al.* (1987) also describe a saran wrap method and an agarose plate method, both of which rely on fluorescence of intercalated ethidium bromide.

The amount of DNA extracted is low by comparison with a similar method of Brikun *et al.* (1994) who reported 30-50 μg of DNA per 0.5 ml of culture compared to approximately 0.4 μg from 5 ml of culture. Extractions from Gram-positive bacteria also had lower than anticipated yields (3-30 μg suggested from 1 ml of culture; Puregene DNA Isolation Kit Instruction booklet). However, the yield is dependent on the number of cells in the culture which is affected by the bacterial strain and growth conditions, as well as genome size and how readily DNA is extracted (Puregene DNA Isolation Kit Instruction booklet) making comparisons between studies troublesome. Yields obtained were similar for all extractions throughout this study.

All extractions used for RAPD-PCR throughout this study were diluted to give a value within the range of 20-100 ng, not only for standardisation but to dilute any residual PCR inhibitors such as SDS and phenol (Roux, 1995).

4.4 RAPD-PCR

The first description of RAPD-PCR was as a technique to study genetic polymorphisms (Welsh and McClelland, 1990; Williams *et al.*, 1990) with amplification profiles displaying intra- and inter-species differences between *Staphylococcus* and *Streptococcus* species (Welsh and McClelland, 1990). Since then it has been applied to many genera of bacteria

including *Lactobacillus* (Johansson *et al.*, 1995), *Listeria* (Farber and Addison, 1994; MacGowan *et al.*, 1993), and *Streptococcus* (Österlund and Engstrand, 1995, Welsh and McClelland, 1990) with varied applications. Applications of the technique have included epidemiological studies of recurrent *S. pyogenes* infections (Österlund and Engstrand, 1995), and hospital outbreaks of *Pseudomonas aeroginosa* (Elaichouni *et al.*, 1994) and *Enterobacter cloacae* (Grattard *et al.*, 1994). Strain typing including strains of *Mycobacterium tuberculosis* (Linton *et al.*, 1994), *Lactobacillus plantarum* (Johansson *et al.*, 1995) and bovine *Leptospira* isolates (Corney *et al.*, 1993), studies of DNA diversity within laboratory derivatives of *E. coli* K-12 (Brikun *et al.*, 1994) and species differentiation of *Listeria* species (Farber and Addison, 1994; MacGowan *et al.*, 1993). Fungal species have also been widely examined with strain typing of *Aspergillus fumigatus* (Aufauvre-Brown *et al.*, 1992) and *Cryptococcus neoformans* (Haynes *et al.*, 1995) and species discrimination of *Candida* (Lehmann *et al.*, 1992). Periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia* and *P. nigrescens* have been studied by RAPD-PCR as described in section 1.8.6.3 of the introduction.

RAPD-PCR is easy to perform, it is also easy to interpret provided that there are sufficiently cultivable strains or species to be used for the comparison of profiles (Niederhauser *et al.*, 1994). The use of RAPD-PCR for typing has been shown to compare favourably with other methods including MLEE (Wang *et al.*, 1993) used to group microorganisms. The transmission of *P. gingivalis* was studied by ribotyping, REA and RAPD-PCR and all three techniques drew the same conclusions (van Steenbergen *et al.*, 1993).

4.4.1 PRIMER CHOICE

The 4 primers used in this study had previously been described by Ménard *et al.* (1992; 970-11), Corney *et al.* (1993; L10 and US) and Welsh *et al.* (1992; RSP), primer details are given in materials and methods section 2.5. Primers L10 and US were used successfully to differentiate serotypes of bovine *Leptospira* isolates (Corney *et al.*, 1993). *Leptospira* are spirochaetes, helical Gram-negative bacteria with axial filaments which cover the cell in the periplasmic space between the peptidoglycan and the cell membrane. The helical form displays variation and *Leptospira* show numerous tight regular coils. Members of this genus are indigenous to animals but can cause a zoonosis in humans. Primer RSP was used for the successful strain differentiation of *B. burgdorferi*, the causative agent of Lymes Disease. *Borrelia*, also spirochaetes, have numerous loose irregular coils and high numbers of periplasmic filaments and are associated with arthropod vector-borne disease. The third genera of spirochaete is

Treponema, members of this genus are found in the oral cavity (Listgarten, 1976; Riviere *et al.*, 1992). The 3 primers, L10, RSP and US were originally chosen for a RAPD-PCR study of oral treponemes (R. Grady, thesis submitted to University of Manchester, 1998) and adopted for this study.

The sequences of primers chosen for RAPD-PCR is arbitrary removing the need to obtain sequence information (Welsh and McClelland, 1990; Williams *et al.*, 1990). In the original RAPD-PCR paper by Welsh and McClelland (1990), the suggestion that primer choice is made simpler if primers in general circulation are used was accepted in this case.

Primer 970-11 was chosen because it had been used to differentiate animal and human strains of *Porphyromonas gingivalis* (Ménard and Mouton, 1993) a periodontal pathogen. This primer is shorter than the other 3 primers (9 bases as opposed to 17, 19 and 20 bases; see materials and methods section 2.5) and this may increase the likelihood of a complementary sequence occurring within the genome (MacGowan *et al.*, 1993).

4.4.2 NEGATIVE CONTROLS

The use of negative controls containing all components of the PCR reaction but with sterile water replacing template DNA is standard practice for both PCR (Roux, 1995) and RAPD-PCR (Farber and Addison, 1994; Linton *et al.*, 1994; Ménard *et al.*, 1992; Meunier and Grimont, 1993 and Myers *et al.*, 1993). No amplification products should be seen within a negative control lane of an agarose gel, confirming that the fragments are due to added template DNA only (Farber and Addison, 1994). However, the contamination of *Taq* and therefore amplification fragments where no template DNA was added has been reported (Meunier and Grimont, 1993). In this case the results were accepted because the bands seen did not match any seen in test samples. In another study (Myers *et al.*, 1993) the use of two particular primers was abandoned due to the appearance of background in the negative control samples. During this study, amplification in the absence of template DNA was seen on only a couple of occasions at which juncture a decision was taken as to whether the bands occurred elsewhere (Meunier and Grimont, 1993).

The arbitrary nature of the primer and low stringency conditions increases the likelihood of any contaminant being amplified and all precautions are taken to avoid contamination.

4.4.3 OPTIMISATION OF RAPD-PCR

Ellsworth *et al.* (1993) describe how banding profiles change with the use of non-optimal conditions with the occurrence of artifactual bands rather than true polymorphic bands. In

the study by Ellsworth *et al.* (1993) particular attention is paid to the concentrations of primer, magnesium and template DNA whilst all other factors are constant. Other researchers (Akopyanz *et al.*, 1992; Muralidharan and Wakeland, 1993; Park and Kohel, 1994; Williams *et al.*, 1993) recognise the need to obtain optimal parameters for these variables. Many other factors including thermal cycler used (Meunier and Grimont, 1993), annealing temperature (Ellsworth *et al.* 1993; Linton *et al.*, 1994; Penner *et al.*, 1993) and transition time between melting and annealing temperatures (Schweder *et al.*, 1995), type of polymerase (Meunier and Grimont, 1993; Schierwater and Ender, 1993), cycle number (Linton *et al.*, 1994), colony age (Coutinho *et al.*, 1993) and method of DNA extraction (Cocconcelli *et al.*, 1995; Linton *et al.*, 1994) have been suggested to affect the reproducibility of amplification profiles and therefore require standardisation.

Within this study, the affect of template DNA concentration and the optimum concentrations of primer and magnesium were determined. The method of DNA extraction including length of growth of cells was standardised, *Taq* polymerase (as well as $MgCl_2$ and buffer IV) were purchased from the same supplier (Advanced Biotechnologies) as were dNTPs (Pharmacia Biotech). The same thermal cycler was used for all analyses. A detailed explanation for adopting these practices is given in section 4.4.4.

4.4.3.1 Magnesium concentration

The efficiency of the amplification reaction is partly dependant upon the magnesium concentration as it is an essential cofactor for the enzymatic activity of DNA polymerases, the concentration is affected by buffer type used and the concentration of deoxynucleotide triphosphates (Roux, 1995) as described in section 1.8.5.2.3 of the introduction. The optimal concentrations of a reaction component is that value at which there is consistent reproducibility of products and a maximum number of bands (Park and Kohel, 1994) and a low amount of background (defined as multiple, indistinct fragments of low intensity by Elaichani *et al.*, 1994).

The optimum value should be determined for each primer under test by means of a titration series in which all other factors are kept constant and a range of magnesium concentrations used (Park and Kohel, 1994). Values between 1.5 mM and 6.0 mM have been suggested (Park and Kohel, 1994), although the use of 7.0 mM has been reported (Li and Caufield, 1998). The need to titrate optimal magnesium concentration for each primer used has been illustrated by Park and Kohel (1994) who tested two primers and demonstrating that the use of 5.0 mM of $MgCl_2$ resulted in a smear and absence of amplification for one primer tested

whilst by contrast, amplification was still occurring at 5.5 mM for a second primer. Despite the necessity of performing magnesium titrations it is discussed infrequently (Cocconcelli *et al.*, 1995; Li and Caufield, 1998) with most studies omitting reference to it (Brikun *et al.*, 1994; Corney *et al.*, 1993; Farber and Addison, 1994; Haynes *et al.*, 1995; Ménard *et al.*, 1992; Myers *et al.*, 1993).

The intensity (Williams *et al.*, 1993) and number (Park and Kohel, 1994) of amplified bands is affected by magnesium concentration. Both an increase (Park and Kohel, 1994) and a decrease (Gao *et al.*, 1996) in the number of bands amplified with increasing $MgCl_2$ concentration have been reported but additionally there is increased amplification (intensity) of some bands and decreased intensity of others (Williams *et al.*, 1993).

4.4.3.1.1 Optimisation of magnesium ions in RAPD-PCR of *P. intermedia* and *P. nigrescens*

A range of magnesium concentrations between 0.5 mM and 5.0 mM were tested for the 4 random primers. Amplification was not seen below 1.5 mM and the use of concentrations below 2 mM have been reported as failing to display amplification products (Cocconcelli *et al.*, 1995). The numbers of amplification products and the intensity of amplification were seen to increase in agreement with observations by Park and Kohel (1994) and Williams *et al.* (1993). At higher magnesium concentrations, new low intensity bands were seen supporting the view of Williams *et al.* (1993) that the intensity of amplification alters however the decreased intensity of previously strong bands was not seen. Ellsworth *et al.* (1993) suggested that above a magnesium concentration of 2 mM amplification occurred independently of the concentration. This view is not shared by Park and Kohel (1994) who suggested the optimum would fall into the range 1.5 mM-4.5 mM, an assumption based on the analysis of 200 primers and other researchers who found values higher than 2mM optimum (Cocconcelli *et al.*, 1995). The optimum values determined in this study, 2.0mM (US) and 3.0mM (RSP, L10 and 970-11), fall within the range of Park and Kohel (1994). Primer 970-11 was previously used for RAPD-PCR of *P. gingivalis* (Ménard *et al.*, 1992) with a $MgCl_2$ concentration of 3 mM equal to the value determined by this study. Primers US, L10 and RSP were reported to be used at with 4 mM $MgCl_2$ (Corney *et al.*, 1993; Welsh *et al.*, 1992) considerably higher than the values determined here, however the differences are probably due to other factors such as the concentration of $MgCl_2$ within the buffer and the concentration of dNTPs (section 4.4.3.1).

4.4.3.2 Primer concentration

The primer anneals to a complementary region of the template DNA and allows *Taq* polymerase to catalyse the production of oligonucleotide chains (see introduction section 1.8.5.2.1). The effect of altering primer concentration on the PCR amplification has been studied in depth by Muralidharan and Wakeland (1993) who found that higher concentration of primer resulted in the amplification of new bands not seen at lower concentrations. If the concentration used is too high smearing as a result of non-specific amplification will be seen (Williams *et al.*, 1993). As with magnesium titrations (section 4.4.3.1), determining the optimal primer concentration although crucial is discussed infrequently (Akopyanz *et al.*, 1992; Li and Caufield, 1998; Linton *et al.*, 1994; Williams *et al.*, 1993) with the majority of studies failing to refer to it (Brikun *et al.*, 1994; Corney *et al.*, 1993; Farber and Addison, 1994; Haynes *et al.*, 1995; Ménard and Mouton, 1993; Ménard *et al.*, 1992; Myers *et al.*, 1993).

4.4.3.2.1 Optimisation of primer concentration for RAPD-PCR of *P. intermedia* and *P. nigrescens*

A range of primer concentrations between 0.5 μM and 5 μM were tested for the 4 random primers. Ellsworth *et al.* (1993) reported that the use of high primer concentrations (6.4 μM) resulted in the amplification of small fragments whilst low primer concentrations (0.1 μM - 0.4 μM) allowed amplification of fragments larger than 500 bp. It was recommended that a concentration within the range of 1.6 and 6.4 μM be used for consistent amplification. These values are considerably higher than the range of 0.1 - 0.2 μM per 25 μl of reaction mix reported elsewhere (Akopyanz *et al.*, 1992; Williams *et al.*, 1993). These discrepancies confirm the necessity of optimising conditions for all primers, neither range of values is considered wrong as it is correct for the conditions under which it was determined.

Primer titration experiments carried out within this study determined the use of 1.0 μM 970-11 and US, 1.5 μM RSP and 2.0 μM L10. These values were higher than the 0.8 μM (50 μl of reaction mix) of 970-11 used by Ménard and Mouton (1993) and Ménard *et al.* (1992) and lower than the 1.0 μM of primer L10 (Corney *et al.*, 1993). Primer RSP was reported as being used at 10 μM in 10 μl of reaction mix (Welsh *et al.*, 1992), therefore the optimum value determined here was considerably lower but as with $MgCl_2$ concentration, it is dependant on other factors which explains the discrepancy. The value of 1.0 μM chosen for primer US was as previously reported (Corney *et al.*, 1993)

4.4.4 REPRODUCIBILITY OF RAPD-PCR AMPLIFICATION PROFILES

The ability of the system to produce reproducible banding patterns was tested in 3 ways for all DNA samples analysed. Experiment variation is guarded against by many researchers who tend to analyse samples in pairs (Akopyanz *et al.*, 1992; Johansson *et al.*, 1995) or triplets (Linton *et al.*, 1994), in this case, intra-experimental reproducibility was checked by analysing samples in pairs. The day-to-day reproducibility has been questioned (Gao *et al.*, 1996; Meunier and Grimont, 1993; Linton *et al.*, 1994) and was checked by repeating all analyses on 3 separate occasions (Meunier and Grimont, 1993). The same thermal cycler was used for all RAPD-PCR analyses in response to reports that the thermal cycler employed for PCR is responsible for reproducibility problems, Meunier and Grimont (1993) reported day-to day reproducibility of results differs between machines with respect to loss and gain of amplified bands. This is probably due to variation in transition time between melting and annealing temperatures (Schweder *et al.*, 1995) with fragments becoming more stable and increasing in number with a longer transition time. The use of the same thermal cycler at all times should have ensured a similar amount of day-to-day variation if any were seen. The same factors probably account for the lack of reproducibility reported between laboratories. Penner *et al.* (1993) demonstrated that even using the same protocol including same primer sequence, under the same laboratory conditions, different size ranges of fragments were amplified.

The ability of the system to produce reproducible patterns between DNA extractions was checked by ensuring that RAPD-PCR patterns were checked for high intensity bands using DNA amplified from at least two separate cultures of the same strain. This has been described by Brikun *et al.* (1994) and Akopyanz *et al.* (1992).

As listed in section 4.4.3, many factors are thought to contribute to the reproducibility and of these, only the affect of template DNA concentration and changing concentrations of primer and magnesium were determined. The method of DNA extraction including length of growth of cells was standardised (the importance of which is described in section 4.4.11). *Taq* polymerase (as well as $MgCl_2$ and buffer IV) were consistently purchased from the same supplier (Advanced Biotechnologies) due to reports that the reproducibility of amplification profiles is affected by the choice of *Taq* polymerase (Meunier and Grimont, 1993; Schierwater and Ender, 1993), therefore enhancing optimised reproducibility if the same enzyme is used at all times (Akopyanz *et al.*, 1992). The use of the same manufacturer of primers is also considered important (Farber and Addison, 1995) although unfortunately this was not possible in the case of L10, whilst previously bought from the

Oligonucleotide Synthesising Service, School of Biological Sciences, University of Manchester, the unit was closed at a time when more was required and an alternative supplier was sought (Perkin-Elmer).

Fluctuations in temperature within the wells of one machine and one temperature profile have been reported (He *et al.*, 1994) where despite the temperature programming, temperatures can fluctuate by as much as 1-2°C between wells and 2-3°C between machines which affects the amplification profile. These temperature differences were more marked for older machines, with negligible temperature differences recorded for newer ones however, a greater temperature variation correlated with lower pattern reproducibility. RAPD-PCR is sensitive to temperature changes because of the low annealing temperatures and short primers used (He *et al.*, 1994). In an attempt to limit this factor as much as possible the wells nearest the temperature probe were always used in the hope they would exhibit the lowest variations in temperature. The problem of well temperature variation is addressed with respect to PCR in materials and methods section 2.8.8, results section 3.11.5 and discussion section 4.6.5, although it must be emphasised that the PCR conditions were different. The affect of annealing temperature was not studied here, however studies indicate that the banding patterns of amplified fragments alters with changes to the annealing temperature (Ellsworth *et al.*, 1993, Williams *et al.*, 1993).

4.4.4.1 Reproducibility of RAPD-PCR amplification profiles of *P. intermedia* and *P. nigrescens*

The concentration of primer and magnesium were optimised for all 4 random primers and the amount of template DNA was approximately equivalent for all reactions (approximate range 20-100 ng, see section 4.3), which may help to eliminate a potential source of reduced reproducibility (Linton *et al.*, 1994) as well as diluting out potential PCR inhibitors (Roux, 1995). Primer L10 was used for the majority of work (material and methods sections 2.5.6-2.5.9) and with the most success.

Linton *et al.* (1994) described that RAPD-PCR typing of *M. tuberculosis* displayed higher intra-experiment reproducibility than inter-experiment reproducibility. The results of this study were not so clear cut, on the whole inter-experiment or day to day variations with primer L10 manifested as slight variations in the intensity of amplified fragments, on no occasions were regularly amplified bands lost and the only times that previously unseen bands were amplified was during the template DNA concentration titration RAPD-PCR (see section 4.4.7) and in months I and II of the DNA amplified at 15 months (stability

experiment; see section 4.4.6). Slight variations in band intensity could be due to either the exact components of the reaction mix (Linton *et al.*, 1994) or the temperature profile of the thermal cycler affected by either the ambient temperature or the location of the sample (He *et al.*, 1994). Intra-experimental reproducibility with primer L10 was excellent, however, on a couple of occasions one of a pair of samples within one experiment would fail to show any amplified products suggesting poor intra-experimental reproducibility. Despite this, amplification was usually successful on another occasion suggesting location of sample within thermal cycler or some other component was at fault. At no time were the marker bands (bands I-IV see section 4.4.8.1) not amplified, although the intensity of band III in *P. nigrescens* ATCC 25261 was occasionally lower than the other strains.

Primer RSP was the least successful, amplifying template DNA for a limited period only. After performing concentration of magnesium and primer titration PCRs as illustrated by figure 3.1 (results section 3.5.2) this primer stopped priming amplification completely. A lack of amplification has been reported by Myers *et al.* (1993) who screened 16 primers for the ability to fingerprint *Haemophilus somnus* isolates and found that one of them gave no amplification of template DNA. No explanation was given but it is probable that either the conditions were not optimised with respect to anyone of a number of factors or the annealing temperature was not sufficiently low to allow primer annealing with mismatches and subsequent amplification. Primers 970-11 and US were not as successful as L10 nor as poor as RSP, both 970-11 and US also failed to prime amplification products at times, with no apparent reason and in all cases, no reaction conditions were altered. The ability of both of these primers to prime amplification appeared to decrease over time and fresh dilutions of primer and template DNA from an extraction that had previously worked well also failed to produce amplification products. He *et al.* (1994) reported, without explanation that some primers are particularly sensitive to environmental conditions, possibly due to extremely inefficient priming, meaning a slight alteration could halt activity. Alternatively degradation of primers and or DNA had occurred, this is considered unlikely as the same templates were used for amplification with primer L10. As explained in section 4.4.3, the success of RAPD-PCR is influenced by many factors which were not altered here.

4.4.5 EFFECT OF RNA ON RAPD-PCR AMPLIFICATION PROFILES

The occurrence of RNA within a DNA preparation was shown not to alter the amplification profile achieved. This is in agreement with work by Ménard *et al.* (1992) who demonstrated that the occurrence of RNA and or protein with DNA did not affect the

amplification profile. In contrast to this result, Ellsworth *et al.* (1993) stated that the presence of RNA may result in artifactual bands that were due to the inaccurate determination of DNA concentration as a result of RNA contamination (see section 4.3 for a discussion concerning methods of DNA quantification).

Despite the lack of need to remove RNA, it is a step commonly employed during DNA extraction by researchers performing RAPD-PCR (Brikun *et al.*, 1994; Corney *et al.*, 1993; Myers *et al.*, 1993).

4.4.6 STABILITY OF EXTRACTED DNA AND ABILITY TO GIVE REPRODUCIBLE AMPLIFICATION PROFILES OVER TIME

In an extended test for reproducibility, DNA from one strain of *P. nigrescens* was extracted every month over 7 months and amplified over 15 months. The reason for this was three-fold, firstly to show that DNA stored for a length of time between 1 and 15 months could be amplified without affecting reproducibility, secondly that a bacterial strain could be continually cultivated within the laboratory between extractions without effect and lastly, to demonstrate that RAPD-PCR was reproducible between DNA extractions.

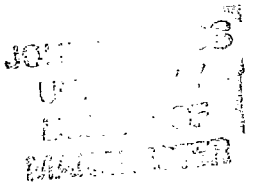
Throughout the 7 months of extractions for this experiment (section 2.5.6; table 2.3) strains were subcultured and maintained in the laboratory as described in materials and methods section 2.3. The lack of significant changes to the banding patterns draws two conclusions. The first is that DNA extracted from *Prevotella* species is stable over time and can be amplified at later dates without loss of reproducibility as all major bands were reproducible with only slight variations in the intensity of amplified bands. This has been reported by Chen and Slots (1995) with DNA from *Actinobacillus actinomycetemcomitans*, *B. forsythus* and *P. gingivalis* when separate PCR reactions were performed 6 months apart and agrees with the findings of Ménard *et al.* (1992) for *P. gingivalis* DNA amplified over 8 months and Preus *et al.* (1993) for DNA extracted from *A. actinomycetemcomitans* and amplified over 6 months. The second conclusion is that the repeated subcutivation of strains within a laboratory does not affect changes in the genomic sequence and therefore no alterations to the banding pattern are seen, this is in agreement with other studies (Elaichani *et al.*, 1994; Gao *et al.*, 1996) including Elaichani *et al.* (1994) were strains were subcultured over two years without affecting the banding pattern.

Extracted DNA is kept at 4°C (Wallace, 1987b; section 4.2.1) to slow the action of internal and environmental DNases which will degrade DNA and affect the banding patterns. The stability of amplification profiles seen here suggest that little degradation is occurring.

4.4.7 EFFECT OF TEMPLATE DNA CONCENTRATION ON RAPD-PCR AMPLIFICATION PROFILES

The present study found that the amount of DNA used did not have a dramatic affect on the results. The relative concentrations of extracted samples were determined by comparison with DNA of known concentrations in an agarose gel. The major bands of high intensity were amplified at all template concentrations, with other bands becoming apparent below 50 ng (200 ng in 50 µl). It has been reported that the use of undiluted DNA in high concentrations resulted in a smear by Williams *et al.* (1993) but in this study, DNA which was undiluted (500 ng) was amplified and displayed discrete bands. The concentration of DNA is not given by Williams *et al.* (1993) but it may have been much higher. In this study, undiluted DNA at the highest concentration resulted in the lowest number of bands, they were also of a lower intensity than seen at other concentrations. This may have been due to the inhibitory affect of components of the DNA extraction procedure (Roux, 1995; section 4.2.1). The range of 250 ng - 5 ng of DNA did not display significant alterations to the banding profiles. Chen and Slots (1995) proposed the use of 10-500 ng of bacterial DNA to give reproducible results. A discrepancy is seen here for the maximum end of the range, during this study 500 ng was not found to amplify as many bands as seen consistently at lower concentrations. Another study reports significant alterations to banding profiles when 200 ng of template DNA is used (Gao *et al.*, 1996). The affect of using concentrations below 1 ng is unclear, studies report that it has no affect (Li and Caufield, 1998) or results in very different patterns (Akopyanz *et al.*, 1992), in this study below 2.5 ng of template DNA amplification of new bands were seen and is not recommended. Williams *et al.* (1993) suggest that reproducible amplification is achieved in most cases with 1 µg/ml template DNA equating with 20 ng in a 50 µl reaction and this was tested with strains of *E. coli*, *S. aureus* and *L. monocytogenes*. This value falls within both the range determined here and that of Chen and Slots (1995). Ellsworth *et al.* (1993) tested template concentrations from 0 µg to 4.8 µg and suggested the range of 0.3 to 1.0 µg (300-1000 ng) as the ideal, which appears quite high compared to Chen and Slots (1995) and Williams *et al.* (1993). However this reinforces the point that optimisation is required for each primer (Ellsworth *et al.*, 1993).

These reports suggest that a wide range of DNA concentrations will successfully produce reproducible banding patterns, however different extraction methods yield DNA of different purities (Williams *et al.*, 1993) therefore it is important to determine the extremes for a



given system. If only the occurrence of major bands are to be noted, then the amount of DNA need not be considered critical, as seen during this study. This summary is in direct conflict with work by Davin-Regli *et al.* (1995) who reported that the reproducibility of RAPD-PCR profiles was significantly affected by this variable. This conclusion was based on concentrations below 100 pg/μl, where bands of high intensity disappeared to be replaced by bands of lower intensity and different sizes. It was suggested that the changes were due to loss of perfect annealing sites or random mismatches (Davin-Regli *et al.*, 1995). A decrease in intensity of some bands with dilution of template although not recorded here, has been reported (Ellsworth *et al.*, 1993).

4.4.8 RAPD-PCR OF *P. INTERMEDIA* AND *P. NIGRESCENS*

4.4.8.1 Random primers 970-11, US and L10

Each of the four primers was used for RAPD-PCR. No amplification patterns other than $MgCl_2$ and primer concentrations were obtained using primer RSP (section 4.4.4.1), whilst the other 3 primers L10, US and 970-11 produced a unique array of patterns for the template DNA. RAPD typing of *P. intermedia* and *P. nigrescens* with primers US and 970-11 revealed considerable inter-species differences as well as intra-species differences. High intensity bands were often seen that were not shared by all strains of a species and fingerprints were unique for each strain tested. This suggests that these two primers are unsuitable for species level identification but could have potential for strain typing. Unfortunately, this could not be substantiated due to the decreased ability of these primers to prime amplification (section 4.4.4.1).

Results with primer L10 exhibited less strain discrimination. Visual analysis of bands suggested two groupings (I and II) which corresponded to *P. nigrescens* and *P. intermedia* respectively confirmed by statistical analysis (section 4.4.8.3.1). Species-specific bands were seen but the *P. intermedia* specific band (IV) at approximately 600 bp was shared between *P. intermedia* and *P. nigrescens*, however the intensity is higher for strains of *P. intermedia* and it occurs with band II at approximately 1500 bp. Despite the obvious similarities between fingerprints of strains, unique polymorphic bands were seen occasionally, for example at 2600 bp in *P. intermedia* MH3. RAPD-PCR has been shown to amplify species-specific bands for other species including *Leptospira* spp. (Letocart *et al.*, 1997), *Mycobacterium bovis* (Rodriguez *et al.*, 1995) and *Porphyromonas gingivalis* (Ménard *et al.*, 1994).

Primer L10 is useful for species typing and affords potential for confirmation of species when used in comparison with known strains of either species.

4.4.8.2 Utility of RAPD-PCR to differentiate *P. intermedia* and *P. nigrescens*

RAPD-PCR analysis is made possible by the fact that different primers generate unique banding patterns for a given DNA template (Sakallah *et al.*, 1995) due to the fact that the occurrence of sequences with some homology to the primer within the genome is random (MacGowan *et al.*, 1993). In addition, the use of low stringency conditions means that mismatches between the primer and the genome are tolerated and amplification is more likely (Welsh and McClelland, 1990). As a result, the usefulness of a particular primer is dependent on the outcome required. Linton *et al.* (1994) tested 40 RAPD primers for the ability to differentiate strains of *M. tuberculosis*, although amplification products were seen with all primers, only 4 were sufficiently discriminatory between strains. In another example, the screening of 200 random primers resulted in 3 primers that offered a distinction between *Listeria* species, strains of the same species and serotypes of strains (Farber and Addison, 1994). Although the number of primers tested here is comparatively small, the effect is illustrated. Primer L10 offers species distinction whilst primers 970-11 and US seem to offer the potential for strain typing, a possibility which was not fully explored (see section 4.4.8.1). The results suggest that screening a larger range of primers is required to find one which provides adequate results for strain typing. The discrimination of isolates at a strain level requires a clear difference in RAPD profiles (Linton *et al.*, 1994), which is not what is seen with primer L10. The use of information from both primers (L10 and another primer which maybe US or 970-11) would provide a comprehensive picture of the DNA diversity among strains of *P. intermedia* and *P. nigrescens*. It has been recommended that a minimum of 3 primers be used to provide an exhaustive RAPD profile (Farber and Addison, 1994) and the routine use of 5 or 6 primers has been suggested (Linton *et al.*, 1994). In the case of *P. intermedia* and *P. nigrescens*, a minimum of two primers would probably be enough, which suggests that the exact number and combination of primers is dependant on the sequence diversity of the test organism and the extent of the information required by the researcher.

RAPD-PCR has recently been used to achieve species differentiation of oral isolates of *P. intermedia* and *P. nigrescens* (Mättö *et al.*, 1996a) using a primer called OPA-13. This primer amplified a 900 bp *P. intermedia* specific band and a 1.3 kb *P. nigrescens* specific band from the majority of isolates. In addition to this, a second primer OPA-03 was used to

study clonal analysis which resulted in 5 unique *P. intermedia* and 17 unique *P. nigrescens* amplification profiles, illustrating the use of more than one primer to provide comprehensive information.

4.4.8.3 Analysis of *P. intermedia* and *P. nigrescens* RAPD-PCR amplification profiles

The bands viewed when RAPD-PCR products are resolved represent DNA polymorphisms due to base deletions or insertions in the genome (Williams *et al.*, 1990). Despite the low stringency conditions, base substitutions may also prevent or allow amplification resulting in a polymorphism (Williams *et al.*, 1990). On a gel, length polymorphisms are indistinguishable from actual polymorphisms due to the presence or absence of a fragment (Welsh *et al.*, 1992) which means scoring bands by their presence or absence may not be appropriate (Welsh *et al.*, 1992). Despite this, the occurrence of a band is usually noted as a polymorphism and the use of visual analysis alone (Linton *et al.*, 1994) or manipulation of binary data (1; present and 0; absent) is commonplace (Ménard and Mouton, 1993). To score bands in this way necessitates the knowledge that bands are not artefacts due to non-optimal conditions (Ellsworth *et al.*, 1993) reinforcing the need for standardisation and optimisation of conditions for each primer to allow reproducible banding patterns and confident band scoring to be undertaken. Analysis of *P. intermedia* and *P. nigrescens* was done by eye (Mättö *et al.*, 1996a) to confirm the appearance of marker bands, this was done during this study and the visual interpretation correlated with cluster analysis.

4.4.8.3.1 Dendrograms; compilation and interpretation

RAPD-PCR bands were sized using a calibration graph determined by the measurement of migration distance of the λ *Pst* I digest fragments plotted against number of base pairs. Amplified bands were sized in all analyses to determine whether bands correlated between gels. Measuring amplified bands in this way is a potential source of error (section 4.4.10). Determining the size of all bands above approximately 600 bp was relatively simple due to clear markers. The 514 bp fragment was usually the smallest maker band seen which allowed adequate size determination of fragments in this size range. However, sizing below this size became progressively more difficult and impossible below 350 bp. It is stressed that all band sizes are approximations.

Bands were scored as present or absent for a binary matrix (section 4.4.8.3). This was determined using 3 repeats of a particular RAPD-PCR so that only reproducible bands were scored. Figures 3.6 and 3.8 illustrate repeated RAPD-PCR amplification profiles. Figure

6.1 is the same as figure 3.6 and has been included to display which bands were scored, although the reader should be aware that some clarity has been lost due to the computer scanning process and low intensity bands are difficult to see.

All dendrograms were produced in the same way after scoring bands amplified by primer L10 with the use of a binary matrix. The simple matching coefficient was used to produce a similarity matrix which was used for cluster analysis by the average linkage between groups or UPGMA (unweighted pair-group) method. The simple matching coefficient was used because it gives equal weighting to a band that is present as to one that is absent.

The 3 dendrograms presented in sections 3.6, 3.7 and 3.9 include increasing amounts of information as the banding profiles of additional organisms was considered. The presence or absence of bands within 7 strains of *P. nigrescens* and 5 strains of *P. intermedia* were scored first (dendrogram 1). The amplification or lack of amplification of these bands was scored for the 4 clinical *P. intermedia* and *P. nigrescens* isolates and the occurrence of amplified bands new to these 4 isolates was studied. Any new amplified bands found within the profiles of the clinical isolates were scored as absent from the known strains. This information became dendrogram 2. Dendrogram 3 was compiled in the same way, taking the information from the previously scored bands of *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 25261 and ATCC 33563 and comparing the profiles of the new species (PINLOs, *P. pallens* and *P. corporis*) for the occurrence of these bands. New bands previously unseen were scored as absent from *P. intermedia* and *P. nigrescens*.

P. intermedia and *P. nigrescens* dendrogram 1

Dendrogram 1 displays two clusters I containing *P. nigrescens* isolates and II containing *P. intermedia* isolates. This confirms the existence of two distinct species as determined by DNA-DNA hybridization (Shah and Gharbia, 1992a) and adds another technique to the list including MLEE (Shah and Gharbia, 1992a, b; Frandsen *et al.*, 1995), SDS-PAGE (Cookson *et al.*, 1996; Gharbia *et al.*, 1994) and ribotyping (Devine *et al.*, 1997; Mättö *et al.*, 1996b) of techniques that will successfully differentiate the species (see section 1.10 for full details). Unique bands represent genomic polymorphisms, therefore when comparing species, these differences indicate genetic heterogeneity and cluster analysis provides evidence for the fact that *P. intermedia* and *P. nigrescens* are highly heterogeneous (Devine *et al.*, 1997; Mättö *et al.*, 1996a). The clustering suggests greater heterogeneity amongst *P. intermedia* compared to *P. nigrescens*, which is in opposition to work by Devine *et al.*

(1997) and Mättö *et al.* (1996a) both of whom suggest greater heterogeneity amongst *P. nigrescens* compared to *P. intermedia.*

***P. intermedia* and *P. nigrescens* clinical isolates dendrogram 2**

Dendrogram 2 includes the 4 black-pigmenting clinical isolates identified as *P. intermedia.* As with dendrogram 1, two major clusters are seen corresponding to *P. nigrescens* (I) and including isolate A and D and *P. intermedia* (cluster II) containing isolate B and C. This result confirms the ability of RAPD-PCR to speciate unknown strains of either *P. intermedia* or *P. nigrescens* when the banding profiles are taken in comparison with those of known strains (see also section 4.17.2.1). Isolates A and D are quite separate from the rest of cluster I and it is possible that they could represent a subspecies of *P. nigrescens.* This is previously unreported, although the possibility of a *P. intermedia* subspecies has been mentioned previously (Mättö *et al.*, 1996b).

RAPD-PCR with primer L10 will differentiate known and unknown strains of the species *P. intermedia* and *P. nigrescens*, both visually and by cluster analysis. The dendrogram shows high levels of intra-species heterogeneity especially amongst strains of *P. intermedia.* Strain typing with either primer 970-11 or US may confirm this. If the limitations of RAPD-PCR are considered and all procedures standardised, it may be considered an attractive method to type the species *P. intermedia* and *P. nigrescens* due to the speed with which it is performed, the lack of molecular information required, the small amounts of DNA needed and the reproducible results.

4.4.9 RAPD-PCR OF PINLOs

The PINLOs used in this study have been shown to be biochemically similar to *P. intermedia* but cannot be differentiated by MLEE and display an alternate pattern of reactivity to monoclonal antibodies (Devine *et al.*, 1994). It was concluded that they represented a new related species (Devine *et al.*, 1994), that were unlikely to be the recently described *P. pallens* (Könönen *et al.*, 1998a). RAPD-PCR was used to compare PINLOs to *P. intermedia* and *P. nigrescens* because of the phenotypic similarities, *P. corporis* was used because it has previously been studied in relation to *P. intermedia* and *P. nigrescens* (Devine *et al.*, 1997) and *P. pallens* because of the recent reclassification of PINLOs (Könönen *et al.*, 1998a, b). RAPD-PCR on this group of organisms was quick and easy because the system had been optimised with respect to $MgCl_2$ and primer concentration.

4.4.9.1 Reproducibility of RAPD-PCR analysis of PINLOs

All PCRs were performed as discussed previously (section 4.4.4) checking for inter- and intra-experimental reproducibility.

On a couple of occasions one of a pair of samples within one experiment would fail to show any amplified products thus demonstrating poor intra-experimental reproducibility. Despite this, amplification was always successful on another occasion suggesting that location of the sample within the thermal cycler may be a factor (He *et al.*, 1994) or inadequate mixing of the reagents had occurred. Slight variations in band intensity were seen between experiments as described, which could be due to either the exact components of the reaction mix (Linton *et al.*, 1994) or the temperature profile of the thermal cycler affected by either the ambient temperature or the location of the sample (He *et al.*, 1994).

4.4.9.2 Analysis of RAPD-PCR amplification profiles

Amplification patterns were very different from *P. intermedia* and *P. nigrescens*, despite some amplification of *P. intermedia* marker bands (see also section 4.18.2.1). Patterns were also different from *P. corporis* and *P. pallens* proving that these strains do not fit into one of the *Prevotella* species studied here. Primer OPA-13 (used for species distinction *of P. intermedia* and *P. nigrescens* by Mättö *et al.*, 1996a) has been used for RAPD-PCR with *P. pallens* strains and amplified a species-specific band. Despite the limited number of PINLOs and comparison species, RAPD-PCR with primer L10 has some potential and further work may result in detection of a PINLO (*P. pallens* or *P. corporis*) marker band. With visual analysis, it shows that these PINLOs and strains of *P. pallens* and *P. corporis* are not *P. intermedia* or *P. nigrescens* confirming the use of primer L10 to distinguish *P. intermedia* and *P. nigrescens*.

4.4.9.3 PINLO, *P. corporis*, *P. intermedia*, *P. nigrescens* and *P. pallens* dendrogram

Dendrogram 3 illustrates the results of RAPD-PCR of the PINLOs compared to other *Prevotella* species. The results confirm the existence of an as yet unclassified PINLOs which cannot be classified as *P. pallens*, by displaying a PINLO cluster which confirms inclusion within the genus *Prevotella* but distinct from other species. Interestingly, the PINLO cluster displays the least similarity to the *P. pallens* cluster. It also displays heterogeneity amongst members of *Prevotella* species and confirms the observation that PINLOs have a low level of heterogeneity amongst strains and that they are distinct from related species (see also section 4.18.2.1).

No major phylogenetic or taxonomic decisions can be based on this dendrogram because it does not contain enough strains of PINLO, *P. corporis* or *P. pallens*.

4.4.10 ERRORS IN RAPD-PCR ANALYSIS

In addition to variations in band intensity, a potential error is introduced when the distances of the fragments are measured and the molecular weights determined between gels (Baleiras Couto, 1995). To try and circumvent this problem or enhance the accuracy, markers were always run on both sides of the test samples, the same gel tray was used and for analyses of the other species PINLO, pallens and corporis, *P. intermedia* and *P. nigrescens* reference strains were incorporated into one gel.

4.4.11 METHOD OF DNA EXTRACTION AND AFFECT ON RAPD-PCR AMPLIFICATION PROFILE

As described in section 4.2.4, many methods of extracting DNA for use in RAPD-PCR have been described. Although this aspect of reproducibility was not covered during this study, it is worth noting. Comparable banding patterns are often described when purified DNA and crude cell extracts produced by boiling are compared (Chen and Slots, 1995; Corney *et al.*, 1993; Elaichouni *et al.*, 1994; Mättö *et al.*, 1996a; Ménard *et al.*, 1992; Welsh and McClelland, 1990) as well as when glass beads are used (Johansson *et al.*, 1995), however these findings are contradicted (Brikun *et al.*, 1994; Johansson *et al.*, 1995). The use of boiled cell lysates reportedly leads to greater variation in band intensity (Corney *et al.*, 1993; Mättö *et al.*, 1996a) and even the occasional loss of fragments larger than 1.8 kb (Ménard *et al.*, 1992), although the end results are as expected when purified DNA is used. DNA extracted using commercial kits has been used for RAPD-PCR and its success is described by Linton *et al.* (1994).

Many extraction methods will work with RAPD-PCR, but as with all other factors it is important to standardise the protocol (Ménard *et al.*, 1992).

4.5 PARTIAL 16S RRNA GENE SEQUENCING

The following section contains a generalised discussion about 16S rRNA sequencing and covers specifically the sequencing of *P. intermedia* and *P. nigrescens* strains, clinical black-pigmenting isolates A-D (see also section 4.17.2.3) and PINLOs (see also section 4.18.2.3). PLS isolates are discussed in section 4.11.11.

Sequencing of 16S rRNA can be performed in several ways and two methodologies are discussed in the following section. The 16S rRNA gene may be sequenced directly using universal primers and reverse transcriptase and although a PCR based technique using universal primers to produce rDNA was undertaken during this study, a brief description of the reverse transcriptase method is provided in the following paragraph (for full details see Lane, 1991). The first step involves extraction and quantification of RNA (methodologies provided in standard texts such as Sambrook *et al.*, 1987) followed by annealing of a primer which primes the synthesis of cDNA by reverse transcriptase (Lane, 1991). Four reactions are run which contain a mixture of dNTPs and a small amount of a particular dideoxynucleotide triphosphate, these prevent chain elongation when incorporated into the new DNA strand (Lane, 1991) as described by Sanger *et al.* (1977). Dideoxynucleotide triphosphates (ddNTPs) are incorporated into a DNA chain by DNA polymerases, but they lack a hydroxyl residue at the 3' position of the deoxyribose. This prevents further chain extension (Sambrook *et al.*, 1987). The result is oligonucleotide fragments of different lengths, which are terminated at every position within the template strand for that particular ddNTP (Sambrook *et al.*, 1987). As one reaction was set up for each ddNTP, each reaction is separated in parallel by electrophoresis and the sequences are read directly from the gel in order of decreasing size of the fragments.

Sequence analysis of rDNA gives a more accurate sequence (Stackebrandt and Liesack, 1993). This is performed by cloning rRNA genes into a vector (this method is not covered in the following section and the reader is referred to standard texts; Sambrook *et al.*, 1987) or commonly, universal primers are used to amplify the 16S rRNA (rDNA) gene which is then sequenced directly (Lane, 1991). The use of direct sequencing of PCR amplified DNA is commonplace (Rao, 1995) and was undertaken during this study by cycle sequencing. This method exploits the temperature profiles of the PCR reaction to denature purified PCR product, anneal primer and extend the product using dNTPs (Rao, 1995) and the dideoxy chain termination technique for DNA sequencing of Sanger *et al.* (1977) as described above.

Although radiolabelled dNTPs can be used (Sambrook *et al.*, 1987), modern studies often use fluorescent dye labelled primers or dNTPs (Kelley, 1994; Rao, 1995). Dye-labelled dNTPs have a specific fluorescent dye associated with a specific nucleotide (Kelley, 1994) and are advantageous compared to dye-labelled primers because the choice of primer is not limited (Chen, 1994). Cycle sequencing occurs as described above, with products extending until a dye-labelled dideoxynucleotide is incorporated (Kelley, 1994).

More than one type of dye-terminator is available (Chen, 1994), although this discussion will cover only ABI PRISM™ BigDye™ Terminator (Perkin Elmer) which was used during this study. The predecessor, ABI PRISM™ terminator cycle sequencing ready reaction mix was also used for a limited period, in an identical way. ABI PRISM™ BigDye™ Terminators are linked to a fluorescin donor dye and a dRhodamine acceptor dye which are brighter than the earlier version and give little background noise when cycle sequencing products are separated (ABI PRISM™ BigDye™ Terminator Protocol). The user obtains an electropherogram which is seen as a series of peaks in which each terminator is represented by a specific colour (A, green; C, blue; G, black and T, red). ABI PRISM™ BigDye™ Terminators give clear peaks (especially G peaks which were often weak with ABI PRISM™ terminators) that are evenly separated.

The type of sequencing analysis conducted during this study was determined by time, this protocol was quick and drew on established techniques with which the author was familiar as well as systems frequently used within the research laboratory.

4.5.1 A SUMMARY OF CYCLE SEQUENCING

As described in section 2.6, this protocol involved 7 steps which are discussed in the following section.

4.5.1.1 PCR of 500 base pair variable fragment from 16S rRNA gene

The first was the PCR of an approximate 500 bp region of rRNA (see section 4.5.5) for a discussion about partial sequencing versus complete rRNA sequencing and section 4.5.2 for discussion about sequencing primers). Sequenced lengths fell into the range of 399 bases (isolate B) to 481 bases (PINLO HST 1156), with an average length of 436 bases.

No attempt was made to standardise the amount of template used in each PCR as this was considered not to be critical. As with RAPD-PCR (section 4.4.2) and specific-PCR (section 4.6.2.1) a negative control was included to check for contamination, and no PCR products were used if amplification was seen in the negative control. In the majority of cases PCR was successful, the only times no product was seen was when some Gram-positive PLS isolates were used as templates and the occurrence of DNA on a gel was negligible. PCR was confirmed by electrophoresis.

4.5.1.2 Purification of PCR product and electrophoresis

This was done using QIAquick PCR purification kit which is suitable for the purification of single and double stranded PCR products which are greater than 100 bp (QIAquick Spin Handbook, 1997) and yields clean templates, free of dNTPs and primer suitable for sequence analysis (QIAquick Spin Handbook, 1997; Rao, 1995). Full details of the purification principle are provided in the QIAquick Spin Handbook (1997) and summarised in the following few sentences. A high salt concentration is used to bind the DNA to a silica membrane contained within a microfuge column whilst all other components of the PCR pass through the column, this is followed by a washing step which removes remaining impurities. The DNA is eluted from the membrane under basic conditions. Buffers are included which provide the correct pH and salt conditions. This step was the most likely point of contamination (Roux, 1995) as any template that is introduced during the post-PCR manipulation phase could potentially be amplified by cycle sequencing (see section 4.5.3). In general if no band was seen after PCR purification, it correlated with a lack of PCR amplification due to insufficient template.

4.5.1.3 Quantification of amount of DNA and cycle sequencing reactions

Only 30-90 ng of purified PCR product are required for cycle sequencing using ABI PRISM™ BigDye Terminators (ABI PRISM™ BigDye™ Terminator Protocol). A discussion about DNA quantification by gel electrophoresis is provided in section 4.3. In this case, the PCR product was compared to a specific fragment (805 bp) of the λ *Pst* I digest. The amount of DNA within this fragment was determined by dividing this by the total number of base pairs within the λ *Pst* I digest (48 502 bp) and multiplying it by the amount seen in the gel (270 ng). This gave approximately 4.48 ng of DNA within the 805 bp fragment. An estimation of the amount of PCR product was made by a subjective estimation of brightness (due to ethidium bromide staining) of the PCR product compared to the 805 bp fragment. As an example, if the 5 µl band of PCR product is 10 times brighter than the 805 bp fragment, this would equal approximately 44.8 ng of DNA in the band (or 8.96 ng/µl), and 5 µl PCR product could be used for cycle sequencing reactions. The amount of DNA was easy to estimate in this way, the PCR product was usually brighter that the 805 bp fragment of λ *Pst* I digest and was diluted as necessary. This approximation was sufficient due to the large concentration range that can be used for cycle sequencing. This is added to the Terminator Ready Reaction Mix which contains dye labelled dideoxynucleotides (A, C, G and T), dNTPs, buffer, $MgCl_2$ and

AmpliTaq DNA polymerase (ABI PRISM™ BigDye™ Terminator Protocol) and requires only the addition of primer and water before use.

4.5.1.4 Ethanol precipitation of products

Cycle sequencing reactions are followed by ethanol precipitation of products which removes excess, unincorporated dye-labelled dideoxynucleotides which would affect analysis (Kelley, 1994). As described in section 2.6.7, sequencing products were separated by Lawrence Hall (University of Manchester) or Oswals DNA service (University of Southampton).

4.5.2 UNIVERSAL PRIMERS

The presence of conserved regions of rRNA means that primers can be designed which will anneal to the majority of species rRNA. Many universal sequencing primers have been described (Lane, 1991), both forward and reverse primers for PCR amplification of the 16S rRNA gene (Lane, 1991) directed to conserved regions of rRNA. These primers are applicable to most eubacteria. In addition, primers have been described to specific groups of organisms (Weisburg *et al.*, 1991) including many human pathogens found in body fluids (Greisen *et al.*, 1994) which included some oral species (*A. israelii, Leuconostoc, Lactococcus, Neisseria* spp., *Streptococcus* spp.) and other pairs have been tested specifically on oral species (Ashimoto *et al.*, 1996; Choi *et al.*, 1994). As a direct consequence of the nature of these primers, contamination of the PCR reaction represents an increased risk as there is a high probability the primers will anneal to the contaminating template and primer amplification. This is in contrast to specific-PCR but similar to RAPD-PCR were the random nature of the primer may have the same affect.

The primers used during this study (forward, RE-TPU1 and reverse, RE-RTU3) were described by Choi *et al.* (1994) for the sequencing of oral spirochaetes by both reverse transcription (RE-RTU3) and PCR (both primers). They were chosen for use in this study because they had recently been used in the same laboratory by Dr. R. Grady (University of Manchester) for some sequencing studies of oral spirochaetes and were therefore available. The occurrence of the primer sequences within *P. intermedia* and *P. nigrescens* was confirmed. There was no difficulty amplifying rDNA using these primers, but if there had have been alternative primers such as those described by Ashimoto *et al.* (1996) could have been employed.

4.5.3 CONTAMINATION OF SEQUENCING PRODUCTS

As described in section 1.8.5.1 of the introduction, any contamination could potentially be amplified. For sequencing this could mean obtaining the sequence of a contaminant organism rather than the one under test. If contamination occurred at the PCR amplification stage, it may be detected by the negative control, otherwise it will result in large amounts of template which will subsequently be purified and sequenced. If contamination occurs during post-PCR manipulations, then there will be a second template available for amplification and sequencing by chain termination. The two species will be in competition with each other and the use of universal primers means that both will contain annealing sites from which to prime amplification. The result is an electropherogram which contains overlapping peaks corresponding to both templates which cannot be resolved and a large number of ambiguous bases. Under these circumstances sequencing is repeated from the beginning. See also section 4.11.11.

4.5.4 ANALYSIS OF SEQUENCE INFORMATION

The electropherogram is a visual representation of the sequence with each base represented by a specific colour (A, green; C, blue; G, black and T, red) the peaks should be evenly spaced, clear and distinct. Ambiguities and unclear sequences were often seen in the first 15-20 bases of the sequence and it has previously been noted (Kelley, 1994) that the first 30 bases of dye terminator cycle sequencing are less accurate than the rest of the sequence. This may include some unincorporated terminators which dominate the sequence at the start (ABI PRISM™ BigDye™ Terminator Protocol). Numerous ambiguities and overlapping peaks may be indicative of contamination (see section 4.5.3).

Dye terminator sequencing has been compared to dye-primer sequencing over a length of 1-500 bases, the accuracy was comparable up to approximately 350 bases after which time dye-terminator dropped from 98% accuracy to 85% (Kelley, 1994). For this reason, it has been suggested that single strand sequences are not used alone to provide information, but that double strands are used for clarification and gap-filling (Kelley, 1994) as was the case in this study. Each strand (forward and reverse) was carefully checked before pairing using the GAP facility. All sequence manipulation during this study was done using the GCG Wisconsin package which runs through a Unix operating system, accessed through Seqnet at Daresbury, UK. The following information is illustrated in appendix 6.8. The first stage was the GAP (section 6.8.3.1) which compares the two sequences; the RE-TPU1 (forward) strand and the reverse and complement of the strand obtained from amplification with RE-

RTU3, this process allows any ambiguous bases to be assigned. Occasionally, there would be very little homology between the two strands, which was taken as an indication of contamination of one and the sequencing was repeated. However, in most cases high levels of homology were seen. Slight differences may be due to incorporation errors of *Taq* polymerase (Fox *et al.*, 1992). Once the sequence is complete, it can be compared with other available sequences. FASTA is used to search and compare DNA or protein sequences (Pearson, 1990; see section 6.8.3.2), it identifies related sequences that share identity with the test sequence (Pearson, 1990). FASTA runs a series of calculations to provide a list of sequence similarity which are explained in detail elsewhere (Pearson, 1990). Although explanation of the FASTA algorithm is outside the scope of this work, a set of 3 numbers is given with each sequences listed by FASTA. These numbers correspond to ***initl***, ***init*** and ***opt*** and an understanding of the derivation of them useful, thus FASTA is summarised in the following sentences. Firstly FASTA finds regions of similarities between sequences and the best regions are rescored to create alignments without gaps, ends are trimmed from sequences so that only bases contributing to the highest score are retained. The highest scoring region is used for the pairwise similarity scoring of pairs of sequences, the initial similarity score (*init1*) is displayed with the search results. FASTA will then check these initial scoring regions and join anywhere possible to create a single alignment and a higher score before calculating an optimal alignment of the initial regions (*initn*). *Initn* scores are used to plot a histogram and calculate mean similarity scores between the test and known sequence before ranking the sequences. The optimised similarity score (*opt*) is determined considering all bases in the initial region (Pearson, 1990). For PLS isolates, after FASTA, no further analysis was done (see sections 4.5.4.3 and 4.11.11). For all other organisms cluster analysis was undertaken. To do this, the sequences were aligned with those from the same species or genus using PILEUP. The alignment of sequences for cluster analysis is affected by insertions and deletions that have lead to a change in length of the molecule (Olsen *et al.*, 1986). As a result of these, the alignment of each base must be considered carefully and if necessary gaps should be introduced to allow alignment of all homologous regions (Olsen *et al.*, 1986). This was done by Seqnet during the pileup procedure and the result is gaps in the sequences which must be removed before distance analysis (illustrated in appendix 6.8.3.3) to allow only the comparison of homologous sequences (Olsen *et al.*, 1986; see section 6.9).

Cluster analyses groups organisms on the basis of similarities between sequences (Olsen *et al.*, 1986; Woese, 1987) and is used frequently for rRNA studies. Whilst there are many

methods used for phylogenetic studies (Olsen *et al.*, 1986), distance matrices were used during this study (section 6.8.3.4). A distance matrix is complied showing the differences (dissimilarities) i.e. the number of point mutations, between pairs of sequences (Olsen *et al.*, 1986). A correction can be included for mutations (Olsen *et al.*, 1986), which in this case was that of Jukes-Cantor (Genhelp). This correction assumes that a nucleic acid substitution can occur at any site with equal probability and that when it does occur, the probability of change to any of the other 3 nucleotides is equal (Genhelp). The distance measurement underestimates true evolutionary distance due to possible multiple mutations occurring at the same point over time and the fact that different parts of a sequence can evolve at different rates (Woese, 1987). The dissimilarity data was not manipulated further but represented visually by means of a dendrogram (section 6.8.3.5). The result is a dendrogram that offers no information regarding genetic distance, only a visual representation of those sequences which are similar.

4.5.4.1 Sequence analysis of *P. intermedia* and *P. nigrescens*

Partial 16S rDNA sequencing of *P. intermedia* and *P. nigrescens* confirms the divisions of the strains as determined by RAPD-PCR. This is also true of the clinical isolates A-D which fall into the correct cluster as determined by RAPD-PCR.

The analysis of partial 16S rDNA sequences displays approximately 91% sequence similarity between *P. intermedia* strains and *P. nigrescens* strains determined over 388 bp. This compares favourably with the figure of 94.7% similarity determined by Paster *et al.* (1994). This value was determined by analysis of 95% of the rRNA gene, therefore is a true reflection of sequence homology. The value of 91% determined by this study reflects the fact that this 388 bp which occur within the first 500 bases of the entire 16S rRNA gene sequence contain significant regions of variability between the species, compared to the remainder of the sequence. The significance of the value determined here is limited by analysis of a partial sequence (section 4.5.5).

Within each species the levels of sequence similarity are equivalent (approximately 98%). Distance calculations (appendix 6.10.1) and dendrogram construction (dendrogram IV, section 3.9.1) suggest a possibility of slightly less similarity between strains of *P. nigrescens* when compared to *P. intermedia*, however no conclusions can be drawn on the basis of this partial analysis.

4.5.4.2 Sequence analysis of PINLOs

Dendrogram V confirms that PINLOs form a distinct species of *Prevotella* compared to the other *Prevotella* species examined here.

The sequences of HST 1156 and HST 2160 are identical over the 441 bases that were aligned and A391 differs from HST 1156 and HST 2160 in only 6 positions at the end of the partial sequence of A391. It may be that a deletion exists within the 16S rRNA of this strain as the sequence here reads GACCGGCT, whereas the other PINLOs, and more importantly all other *Prevotella* have a sequence which reads GGACCGGCT (note that this terminal T is not included within the trimmed sequence but is the next base of the unedited sequences). The only difference being a pair of G bases at the start of this segment. Alternatively, there may have been an error in sequencing or reading the sequence. If either of these possibilities are true, and these 441 bases of the PINLOs 16S rRNA gene are identical, then complete 16S rRNA gene sequencing will be required to elucidate any differences.

Within the PINLOs, a level of approximately 98% sequence similarity was observed, although HST 1156 and HST 2160 displayed 100% identity. This is equivalent to the level of identity seen within strains of both *P. intermedia* and *P. nigrescens* which suggests that the 3 PINLOs may be grouped together. The least sequence distance is seen between the PINLO strains and *P. corporis* (approximately 94% similarity), suggesting a close but distinct relationship. This is reinforced by RAPD-PCR which results in amplification profiles containing bands seen in *P. corporis*, *P. intermedia* and *P. nigrescens* strains. Despite the name PINLO and the biochemical identity to *P. intermedia* and *P. nigrescens* the distance between the partial sequences of these species and PINLO is larger than when compared with *P. corporis*. This is also true of *P. pallens* and suggests the term PINLO should still be employed to cover other uncharacterised organisms.

These findings are not challenged when the information in dendrograms IV and V are amalgamated.

4.5.4.3 Sequence analysis of PLS isolates

Partial 16S rDNA sequencing of PLS isolates consisted only of checking the sequences and performing FASTA searches. Sequence analysis was used in this case as an identification tool for isolates. Distance calculations were performed which confirmed the FASTA results but the values were not used further (distance matrices can be found in appendicies 6.10.3.1-6.10.3.6).

4.5.5 PARTIAL 16S RRNA SEQUENCING VERSUS COMPLETE 16S RRNA SEQUENCING

This study used only partial sequences with lengths within the range 399 bases to 481 bases, for species comparisons. Amongst strains of *P. intermedia* and *P. nigrescens*, the majority of sequence variability is constant between strains of a species, in other words, all strains of *P. intermedia* contain one bases whilst all strains of *P. nigrescens* have another. This is described in section 3.9.1 of the results and the 24 positions of sequence heterogeneity are labelled a-z in section 6.9.1. In addition to these apparently species-specific regions, there are 6 positions (18; 175; 203; 379; 380; 386) with no consensus sequence between the strains within one of the species. These are shown in bold in the sequence alignment in appendix 6.9.1 and consist of one or more strain containing an alternative base. Only repeat sequence analysis will determine whether these reflect true strain variable sites or sequence determining errors. However, the presence of species-specific sites confirms the utility of partial 16S rDNA sequence analysis for these species. As a result of the use of only partial sequences, no comment about phylogenetic relationships between the species examined can be made (Ludwig and Schleifer, 1994; Stackebrandt and Goebel, 1994). However, the partial sequences are adequate for identification purposes (Ludwig and Schleifer, 1994) within the limits of sequences differences. Over the entire length of the 16S rRNA approximately 97% is considered a species relationship (Stackebrandt and Goebel, 1994). All studies which use 16S rRNA sequencing techniques do so to elucidate phylogenetic relationships and therefore sequence the full length. Partial sequences have been reported in studies (Benlloch *et al.*, 1995; Choi *et al.*, 1994) that were used to illustrate sequence diversity and many partial sequences are contained within the databases of EMBL and Genbank.

Partial 16S rRNA gene sequencing was used for two reasons, the first that the PCR primers were readily available and being successfully used. The second was the speed and convenience. The idea was that the variable regions would provide the most information about the differences between closely related species (Busse *et al.*, 1996) such as *P. intermedia* and *P. nigrescens* that had previously been classified by alternative methods and this could be compared with the information already available, which is extensive.

4.5.6 16S RRNA SEQUENCING AND THE SPECIES CONCEPT IN PROKARYOTES

The definition of a species within microbiology is a subject of debate (for information see Fox *et al.*, 1992; Olsen *et al.*, 1986; Stackebrandt and Goebel, 1994; Ward, 1998) concerning whether or not species as defined by the bacteriologist represent natural

divisions (Stackebrandt and Goebel, 1994), based on evolutionary history (Ward, 1998) with differences between nucleic acid or gene sequences reflecting evolutionary divergence (Ludwig and Schleifer, 1994). The use of DNA-DNA hybridization is accepted as the method by which species relationships are determined (Wayne *et al.*, 1987) with the accepted value for DNA similarity defined as 70% or greater with a difference in T_m of 5°C or less (Wayne *et al.*, 1987). The use of sequencing techniques to compare the 16S rRNA genes of organisms has become a corner stone in the comparison of species (Stackebrandt and Goebel, 1994). Whilst a species which have DNA similarity values of 70% or more often have a sequence similarity of at least 97% and are therefore related at the species level (Stackebrandt and Goebel, 1994), it is not always true. Almost identical 16S rRNA sequences have been seen between organisms with less than 70% DNA similarity by hybridization which suggests separate species (Fox *et al.*, 1992). It is suggested that 16S rRNA sequence analysis does not always agree with hybridization data which is still considered a standard (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). 16S rRNA sequence information is not sufficient for classification purposes alone, but of great value in a polyphasic approach (Stackebrandt and Goebel, 1994) which utilises phenotypic and genotypic characters (Ward, 1998). As a result a threshold value for species definition by 16S rRNA homology is not available, but the term rRNA species complex has been coined for those strains which exhibit identical rRNA sequences but DNA-DNA homology levels between 30 and 70% (Fox *et al.*, 1992). The technique should be considered of value for determining strain identities once information regarding the relevant species has been collected (Fox *et al.*, 1992), as the use of sequence comparisons to elucidate relationships is directly affected by the number of organisms studied in this manner (Stackebrandt and Goebel, 1994).

Whilst differences between 16S rRNA sequences are concentrated mainly in hypervariable regions, study of these alone or other segments of the 16S rRNA is not statistically desirable and will therefore influence grouping of organisms within phylogenetic trees (Ludwig and Schleifer, 1994; Stackebrandt and Goebel, 1994) therefore, phylogenetic studies should always be conducted using complete sequences (Ludwig and Schleifer, 1994). The conserved regions are useful for sequence alignment (Ludwig and Schleifer, 1994).

Phylogenetic studies based on 16S rRNA sequences have resulted in alterations to bacterial taxonomy, the influence of this on the taxonomy of oral bacteria is discussed by Tanner *et al.* (1994).

4.6 PCR

4.6.1 DESIGN OF SPECIES-SPECIFIC OLIGONUCLEOTIDES

4.6.1.1 Target for PCR and use of 16S rRNA sequence databases for selection of primer sequences

Targets for PCR reactions include enterobacterial repetitive intergenic consensus (ERIC) sequences found in Gram-negative bacteria, which have been used to type strains of *Serratia marcescens* (Liu *et al.*, 1994) and specific genes such as the leukotoxin gene of *A. actinomycetemcomitans* (Watanabe and Frommel, 1996) and the fimbrial gene of *Porphyromonas gingivalis* (Watanabe and Frommel, 1996). The 16S rRNA gene is a frequently used target for PCR of periodontal organisms (Slots *et al.*, 1995) and the one chosen for this study. The reasons it is so widely used are covered in detail in section 1.8.8.3 and summarised here. The 16S rRNA has a conserved function and is therefore present in all organisms and is composed of species conserved regions interspaced with highly variable sequences unique to the genome which differ between closely related species (Stackebrandt and Liesack, 1993). It is therefore possible to find regions unique to a species (Slots *et al.*, 1995). In addition to this, 16S rRNA gene sequences for many organisms are readily assessable (EMBL, Genbank) including many putative periodontal pathogens and related species. Those organisms (listed in table 6.3, appendix 6.11) chosen for the comparison of potential primer sequences were accepted periodontal pathogens as well as other organisms found within the oral cavity and *Prevotella* species.

4.6.1.2 Choice of sequences

A PCR primer should be specific, amplifying the desired template as proved by separation of products by electrophoresis (Dieffenbach *et al.*, 1993) and in conventional PCR, only one product should be seen. The rules concerning primer design are detailed in section 1.8.5.2.1 and apply to primers for any form of PCR. The sequences chosen were in regions were there was little homology between the consensus sequence of *P. intermedia* and *P. nigrescens*. There were several regions were there was a lack of homology between the species that could have been exploited, the majority were within the first 900 bp of the sequence. Not all the potential sequences were studied and this would be corrected in future studies.

Only the length of the primer was considered during the initial stages. At the time only one of a primer pair was being designed and complementarity to, and T_m of primer RE-TPU1 was not considered until much later. Sequences were gradually discarded as the

complementarity to other organisms and the ability to form stemloop structures was considered. The design of these primers was not optimal, there were more sequences that could have been analysed, in particular the region between approximate base numbers 185 and 240 from which 6 potential sequences were chosen. The analysis should have included some determination of theoretical melting temperature, study of the 3-prime end and greater consideration of the other primer of the pair.

4.6.1.3 Final choice of sequences and RE-TPU1

Only after two sequences (P-int and P-nig) were chosen was the other primer considered. The length of the sequence to be amplified is not critical, somewhere in the range 150-1000 bp has been suggested (Dieffenbach *et al.*, 1993) and with this in mind the position of two readily available universal primers in relation to P-int and P-nig was examined. Primer RE-TPU1 is the forward primer (5-prime to 3-prime) used for PCR based sequence analysis (section 4.5.2), this meant that the reverse direction and complement of the *P. intermedia* or *P. nigrescens* sequence was required for amplification to take place. The annealing positions of RE-TPU1 and the second primer were known, therefore approximate product sizes determined (204 bp P-int; 228 bp P-nig; 499 bp 1Bi-1; 488 bp 2Bi-1). The sequence alignment displayed in appendix 6.12 shows the annealing positions of the primers. RE-TPU1 was chosen as the forward primer for PCR reactions because it was in use within the laboratory and known to amplify DNA from *Prevotella* species in combination with another universal primer, however, a second primer could have been designed either up or downstream and used in its place. Alternatively a previously described primer (section 4.5.2) would have been suitable. RE-TPU1 is a universal primer and thus not specific, specificity of the reaction is therefore dependent on the species-specific primer, especially if the system were to be employed in a clinical environment. The use of a species-specific 16S rRNA directed primer plus a forward universal primer has been reported satisfactorily (Conrads *et al.*, 1996). The use of only one specific primer may be advantageous, it is cheaper and offers flexibility of use such as hybridization (see section 4.20.5.1).

Primers P-int and P-nig were compared to sequences 1Bi-1 and 2Bi-1 which were also used as PCR primers with RE-TPU1, but the sequences could have been reoriented and used together (P-int with 1Bi-1). It is clear that choosing the second of the primer pair is as important and potentially troublesome as choosing the first and how in this case the best options were possibly not explored.

4.6.1.4 Published *P. intermedia* and *P. nigrescens* specific oligonucleotide sequences

Sequences 1Bi-1 and 2Bi-1 (Dix *et al.*, 1990) were chosen because they have been frequently used and successful (Conrads *et al.*, 1996; Mättö *et al.*, 1996b; Shah *et al.*, 1995). The use of the two sequences as nucleic acid probes has been confirmed (Mättö *et al.*, 1996b; Shah *et al.*, 1995) when tested against a range of *Prevotella* sp. and oral isolates representing 32 species or genera (Mättö *et al.*, 1996b) including *Bacteroides* sp., *Bilophila* sp., *Capnocytophaga* sp. and *Porphyromonas* sp. Both 1Bi-1 and 2Bi-1 were specific, although some cross reactivity was reported between the species (Mättö *et al.*, 1996b; Shah *et al.*, 1995), with 1Bi-1 detecting both *P. intermedia* and *P. nigrescens* under conditions of low stringency (Shah *et al.*, 1995). The two sequences were also used as reversed primers as one of a pair with a universal forward primer for PCR species differentiation (Conrads *et al.*, 1996; Conrads *et al.*, 1997) of organisms within plaque and successfully identified *P. nigrescens*.

Dix *et al.* (1990) described five *P. intermedia* and two *P. nigrescens* specific sequences, including 1Bi-1 and 2Bi-1. None of the others were considered in this study on the basis of work by Shah *et al.* (1995) which determined the suitability of the sequences to act as species-specific probes.

Other sequences have been reported, Ashimoto *et al.* (1996) described *P. intermedia* and *P. nigrescens* specific primer pairs and Slots *et al.* (1995) described an oligonucleotide primer pair specific to *P. intermedia*.

4.6.2 USE OF SPECIES-SPECIFIC OLIGONUCLEOTIDES AS PCR PRIMERS

4.6.2.1 PCR

In this study the species-specific oligonucleotides were used as PCR primers. As an alternative, they could have been used as oligonucleotide probes (see section 4.20.5.1). It was decided to use these oligonucleotides as PCR primers because the other techniques used within this research were PCR based techniques and therefore it was decided that PCR with species-specific primers would complement the other methods.

Qiagen was chosen as the supplier of *Taq* for these experiments. This was chosen because the buffer supplied with the *Taq* contains K^+ and NH_4^+ ions, which often allow for less critical magnesium concentration optimisation and a larger annealing temperature range. This is achieved through the combination of cations. K^+ stabilises primer annealing by binding to the phosphate groups of the DNA and NH_4^+ interacts with hydrogen bonds between bases and will destabilise the weak ones (i.e. mismatches). As a result, the buffer

helps to maintain specific annealing even at lower temperatures when non-specific annealing predominates (Löffert *et al.*, 1997). Magnesium ions help to stabilise primer and template interactions, the presence of NH_4^+ destabilising non-specific interactions means that specific annealing is more likely to occur over a range of Mg^{2+} concentrations (Löffert *et al.*, 1997). Despite this, optimisation experiments were still performed (section 4.6.2.2) due to the high amount of non-specific annealing seen at first.

Initial experiments were performed using only the type strains of each species (*P. intermedia* ATCC 25611, *P. nigrescens* ATCC 25261 and *P. nigrescens* ATCC 33563). This seemed a sensible way to test for specific amplification, owing to the fact that no major 16S rRNA sequence differences were apparent in the regions of the primers. However in the light of later results (section 4.6.7) which showed inconsistent and selective amplification of the correct species, all *P. intermedia* and *P. nigrescens* strains could have been tested at the end of each type of optimisation experiment, i.e. when a 'specific' annealing temperature was reached and after the optimal Mg^{2+} concentration was determined. This might have helped pinpoint the aspect of the PCR reaction requiring further work to obtain species-specificity.

The negative controls were suitable and sensible (*A. actinomycetemcomitans*, *C. ochracea* and *P. gingivalis*), although an alternative *Prevotella* species (e.g. *P. corporis*) could have been included. This would have altered the optimal conditions of primer P-nig which amplified DNA from *P. corporis* (see section 3.11.3.2) before the specificity was tested against large numbers of species.

An alternate size marker (ϕx174 DNA - *Hae* III digest) was used when PCR products were resolved by electrophoresis. This was due to the fact that the PCR amplified fragments were small (less than 500 bp, see section 2.8.4) and that the smallest fragment routinely seen by ethidium bromide staining of a λ *Pst* I digest was 514 bp. ϕx174 DNA - *Hae* III digest yields fragments in the range of 1 353 bp to 72 bp, thus comparison of fragment and amplified band is easier.

4.6.2.2 Optimisation of PCR reaction

The factors affecting the PCR are discussed in section 4.4.3 with reference to RAPD-PCR, and apply here for optimising PCR conditions especially the concentration of magnesium ions and annealing temperature (Roux, 1995). Magnesium concentration is discussed in

detail in section 4.4.3.1 and will not be covered here and primer concentration is mentioned only briefly.

The approach taken to optimising the PCR reaction involved annealing temperature, magnesium concentration and finally primer concentration. In this case a temperature cycle that was known to work with one of the primers (RE-TPU1; 57°C, see section 2.6.1) was used. No attempt was made initially to determine approximate T_m for either primer.

4.6.2.2.1 Annealing temperature

The starting point for annealing temperature optimisation was that at which the universal primers were known to prime successful amplification. An alternative starting point is to determine the theoretical melting point (T_m) defined as the point at which double stranded DNA (primer/template duplex) dissociates to single stranded (Dieffenbach *et al.*, 1993) and use an annealing temperature within a couple of degrees of this (Dieffenbach *et al.*, 1993). Several equations are available for determining the T_m. Equation 1 is considered suitable for primers of 20 bases or less (Dieffenbach *et al.*, 1993) and equation 2 will determine the theoretical T_m for oligonucleotides of up to 70 nucleotides (Sambrook *et al.*, 1987). All values are approximations (Roux, 1995).

Equation 1: $T_m = 4 (G + C) + 2 (A + T)$

Equation 2: $T_m = 81.5 + 16.6 \times \log[Na^+] + 0.41 \times (\%GC) - 600/l$

(where l = number of bases and Na^+ is in molarity (M)).

The theoretical T_m of each primer is compared with the annealing temperature that was used in table 4.1. The annealing temperatures used for PCR were higher than the theoretical temperatures. This may help to account for the fact that amplification of all *P. intermedia* or all *P. nigrescens* strains was not seen with the relevant specific primer. However, determining the annealing temperature in this manner is empirical and is only useful as a starting point for a new pair of primers (Roux, 1995), although a starting temperature of 37°C has been suggested for new primer pairs (Williams, 1989). In reality, the annealing temperature is affected by all buffer components as well as concentrations of primer and template (Roux, 1995). The values determined here (table 4.1) are incorrect insofar as the supplier of the PCR buffer (Qiagen) do not reveal the concentration of salt (in this case potassium ions) in the buffer. Therefore the value of 50 mM was chosen based on the

presence of both KCL and NH_4SO_4 (see section 4.6.2.1) in the buffer and the components of Buffer IV (Advanced Biotechnolgies; section 2.2). The components of the Qiagen PCR buffer should remove the need to optimise annealing temperature (Löffert *et al.*, 1997) and therefore a large difference between the T_m and the actual temperature used may be seen. Each primer of the pair should be designed to have a similar T_m (Dieffenbach *et al.*, 1993; Roux, 1995). If this is not the case, then the T_m used should be at the level of the lowest one to allow it to anneal, although this will encourage mispriming by the other one (Dieffenbach *et al.*, 1993). In this case, the annealing temperature of RE-TPU1 was only 2.5°C lower than P-nig, but 7.1°C lower that P-int and 2-Bi1. It has been noted that primers between 18 and 24 bases long will often anneal specifically even if the temperature is not at the optimum (Dieffenbach *et al.*, 1993) which accounts for the amplification seen with these poorly matched pairs.

Primer annealing usually takes place within the range of 37-55°C (Dieffenbach *et al.*, 1993), therefore the temperatures used here are higher than average. This is either due to the true specificity of these reactions at these elevated temperatures or the Qiagen PCR buffer (Löffert *et al.*, 1997).

4.6.2.2.2 Primer concentration

It has been reported that concentrations in the range 0.05 μM -0.5 μM should work for most primers (Saiki,1989), and the value seen here falls within this range.

Table 4.1 PCR primers; theoretical and actual annealing temperatures

	THEORETICAL T_M (°C)	ANNEALING TEMPERATURE USED (°C)
P-int	55	67
P-nig	50.8	65
1Bi-1	53.7	68
2Bi-1	55.4	69
RE-TPU1	48.3	57-69
RE-RTU3	51.6	57

4.6.3 AMOUNT OF DNA TEMPLATE / SENSITIVITY OF PCR

During this study, all optimisation experiments were done with approximately equivalent amounts of DNA (Watanabe and Frommel, 1996).

For PCR detection in clinical studies, the lower limit of DNA required is often quantified in terms of number of cells or colony forming units (Ashimoto *et al.*, 1996; Watanabe and Frommel, 1996). PCR of periodontal pathogens typically reports 10-100 cells (Ashimoto *et al.*, 1996; Conrads *et al.*, 1997; Watanabe and Frommel, 1996). This study confirmed that the PCR reaction would detect approximately 20 ng of DNA however no work was done to relate that to the number of cells or to determine whether less than 20 ng could be detected. In the case of primers P-nig and 2Bi-1 especially, further optimisation is required before this could be done. The number of bacteria in a culture for DNA extraction could have been determined simply by counting the number of cells using a haemocytometer in a known amount of broth (e.g. 10 µl) and using this to estimate the total number of cells (Watanabe and Frommel, 1996). This is then correlated with the concentration of DNA determined spectrophotometrically (Watanabe and Frommel, 1996) or by other methods (section 4.3).

After PCR amplification it is possible to semi-quantify the amount of bacteria in each positive sample by using a dot-blot hybridization assay and the species-specific primers as 16S rRNA directed DNA probes (Conrads *et al.*, 1996; Conrads *et al.*, 1997). The hybridization signal intensity is compared to that for a known number of cells and the equivalent DNA concentration (Conrads *et al.*, 1996).

4.6.4 MULTIPLEX PCR

Multiplex PCR uses multiple primer pairs in combination to amplify more than one target and give multiple PCR products. Primers which anneal to template common in all test samples serve as positive controls for amplification (Edwards and Gibbs, 1995). In this case, the universal primers RE-TPU1 and RE-RTU3, perform this function. Multiplex PCR may display preferential amplification of the shortest fragment where lengths differ but they share some sequence identity (Edwards and Gibbs, 1995) in other words competition occurs. Ideally, amplification occurs due to both primer pairs, otherwise only product from the most favourable primer pair is seen.

Multiplex PCR was used here to examine the amplification efficiency of primers. This was done by comparing the annealing of the specific primers at the optimal conditions determined previously, with amplification by the universal primers which were known to

amplify the template (section 2.6.1), to determine which primer pair was most favourable. No attempt was made to optimise multiplex PCR.

A lack of complementarity between primer pairs is an important consideration for multiplex PCR (Dieffenbach *et al.*, 1993) and alternative primer interactions could account for the third band seen in figure 3.16. Ideally, all primers should exhibit similar melting temperatures (Edwards and Gibbs, 1995). For the detection of multiple fragments, each primer pair should be tested individually, before combining and optimising conditions (Edwards and Gibbs, 1995). Equimolar PCR primer concentrations are not necessary for PCR (Edwards and Gibbs, 1995), as a result, the amount of universal primer RE-RTU3 was halved to determine whether the annealing of these primers was favourable even at reduced concentrations. This was the case with primers P-nig and 2Bi-1.

The results suggested that the amplification with primers P-int and RE-TPU1 and 1Bi-1 and RE-TPU1 was more favourable than RE-TPU1 and RE-RTU3, however the opposite was true for P-nig and 2Bi-1, reinforcing the fact that they are either poor sequence choices, incorrectly paired or not used at optimal conditions.

Use of a primer pair to *P. intermedia* and one to *P. nigrescens* within a multiplex PCR would allow the simultaneous detection of the two species, using only one PCR reaction, and therefore minimising reagents and costs (Edwards and Gibbs, 1995). If this were performed with a clinical sample, universal bacterial primers would confirm that the system was working by amplifying DNA from any organism present. The inclusion of a *Prevotella* sp. (*P. intermedia* and *P. nigrescens*) primer pair would be preferable. It would also provide a rapid method of identifying cultured isolates. The use of multiplex primers to 16S rRNA for species identification has been successfully demonstrated for the Gram-positive aerobic rod *Nocardioides* (Park *et al.*, 1998).

This idea has been expanded allowing the simultaneous detection of 3 periodontal pathogens; *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*, which actually included *P. intermedia* and *P. nigrescens* using primers designed to the 16S rRNA (García *et al.*, 1998).

4.6.5 LOCATION WITHIN THE THERMAL CYCLER

Hoelzel (1990) studied the temperature profiles of various types of thermal cyclers and found that the design of the thermal cycler heating and cooling elements and the location of the temperature thermocouple greatly affected how closely the actual temperature profile resembled the programmed one. The thermal cycler used during this study is heated by a

halogen lamp and cooled by air forced out by fans. The location of the thermocouple within the block determines the temperature profile and temperature can be markedly different at opposite sides across the location of the fan, meaning that some wells will never attain the correct temperature. This can be compensated for by selecting the wells that are used (Hoezel, 1990). This feature helps to account for inconsistencies in amplification seen when the primers were tested.

4.6.6 APPLICATION OF PCR DETECTION OF CERTAIN PERIODONTAL MICROORGANISMS

The use of PCR to detect know nucleic acid sequences from specific bacterial pathogens (Peter, 1991) within clinical samples is commonplace. The technique is being exploited for the detection of periodontal pathogens within plaque to help determine the pathogenesis of periodontal disease, identify those at risk of developing disease or to determine the success of treatment (Watanabe and Frommel, 1996). PCR offers a fast, sensitive and reproducible detection system that does not necessitate purified DNA unlike RAPD-PCR (Watanabe and Frommel, 1996) and does not rely on the ability to cultivate an organism (Peter, 1991). Optimised PCR will detect low, possibly subclincal levels of an organism (Peter, 1991; Watanabe and Frommel, 1996), as low as 10-100 cells has been described (Watanabe and Frommel, 1996). *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola* (Ashimoto *et al.*, 1996; Slots *et al.*, 1995; Watanabe and Frommel, 1996) *B. forsythus*, *Campylobacter rectus*, *E. corrodens* (Ashimoto *et al.*, 1996; Slots *et al.*, 1995), *P. intermedia* (Slots *et al.*, 1995) and *P. nigrescens* (Ashimoto *et al.*, 1996) have all been detected by PCR.

4.6.6.1 Application of PCR to detect *P. intermedia* and *P. nigrescens*

It has been suggested that if there is only a few base differences between closely related organisms, separation by 16S rRNA sequence based PCR will not be possible (Slots *et al.*, 1995). Despite the similarity between *P. intermedia* and *P. nigrescens*, detection of as few as 25 colony forming units of these species by PCR has been achieved (Ashimoto *et al.*, 1996). Other successful primers have also been described (Conrads *et al.*, 1996).

4.6.7 SPECIFICITY OF PCR REACTION

Although the *P. intermedia* primer (P-int) did not amplify DNA from other *Prevotella* species or oral species, they did not amplify DNA from all *P. intermedia* strains. Examination of the sequence alignment offers no clear explanation for this (figure 4.1). Note that the sequences used as PCR primers were the reverse and complement (section

4.6.1.3) of those in figure 4.1 (P-int) and 4.2 (P-nig). With the exception of a couple of international base codes (appendix 6.5), there are no differences between the 16S rDNA sequences of the *P. intermedia* strains which could explain failure to prime amplification. It is possible that these inconsistencies would be resolved with further optimisation experiments (Roux, 1995) or that the DNA template had been degraded over time resulting in the loss of this annealing site. Extracting fresh DNA from those strains not amplified (*P. intermedia* MH6, MH12 and MH15) would check this. As with P-int, amplification of *P. nigrescens* strains with P-nig was not consistent for all *P. nigrescens* strains and other *Prevotella* species were also amplified. Closer examination of the 16S rRNA sequence alignment revealed an area of 6 bases that was removed from the sequence alignment before distance analysis (section 4.5.4) due to the uncertainty over the origin of the bases. Figure 4.2 (a) illustrates the sequence alignment and the region that was removed is shown in bold. It must be noted that this region can be altered in more than one way to produce a consensus sequences for *P. nigrescens* (figure 4.2 (b)). The uncertainty surrounding this region may contribute to the inconsistencies seen during PCR with this primer. It is unclear why P-nig should amplify only *P. intermedia* strains MH3 and MH15, when the sequences of all *P. intermedia* strains in this region are identical. Amplification of PINLO strain HST 1156, *P. pallens* and *P. corporis* was also seen and there is some sequence identity between the 16S rDNA sequence of these species and P-nig, (figure 4.3) which may allow annealing at below optimal temperatures. It is of note however, that there is no difference between the 16S rDNA sequences of PINLO strain 1156 and 2160 in this area and strain HST 2160 was not amplified.

Neither 1Bi-1 nor 2Bi-1 were tested against other strains and species. Both primers fall at the limit of the partial sequences determined for *P. intermedia* and *P. nigrescens* strains and thus no suggestions can be made regarding their success. The use of *P. nigrescens* primer 2Bi-1 was abandoned on the basis of the inconsistent amplification seen during DNA detection limit experiments and on the results of the multiplex PCR, therefore it was not tested against other species, as a poor performance was expected.

Figure 4.1 Sequence alignment of *P. intermedia* primer P-int and other *P. intermedia* strains

```
P-int              TG TCCACATATG GCATCTGACG TG

Pi6                TG TCCACATATG GCATCTGACG TG
Pi12               TG TCCACATATG GCATCTGACG TG
Pi3                TG TCCACATATG GCATCTGACG TG
2 3A       (B)     TG TCCACATATG GCATCTGASG KG
Pi ATCC 25611      TG TCCACATATG GCATCTGACG TG
5 1D       (C)     TG TCCACATATG GCATCTGACG TG
Pi15               TG TCCACATATG GCATCTGACG TG
Pi ATCC 25611      TG TCCACATATG GCATCTGACG TG
```

KEY:

*Pi*3, *P. intermedia* MH3; *Pi*6, *P. intermedia* MH6; *Pi*12, *P. intermedia* MH12; *Pi*15, *P. intermedia* MH15; *Pi* ATCC 25611, *P. intermedia* ATCC 25611; **1** *2B* (A), PLS isolate *P. nigrescens*; **2** *3A* (B), PLS isolate *P. intermedia;* **5** *1D* (C), PLS isolate *P. intermedia*; **3** *2A* (D), PLS isolate *P. nigrescens*

Figure 4.2 Two possible sequence alignments of the region of 16S rRNA chosen as *P. nigrescens* primer P-nig

The alignment displayed in (a) was used during distance analysis and the bases in bold represents positions that was removed. Alignment (b) represents an alternative.

(a)

P-nig	CAAAG GTTTTCCGG TAAGGGA
Pn ATCC 33563	CAAAG G.**TTTTC**CGG TAAGGGA
Pn ATCC 25261	CAAAG G.**TNTTC**CGG TAAGGGA
*Pn*5	CAAAG G**TTTTTC**CGG TAAGGGA
*Pn*94	CAAAG G**TTTTYC**CGG TAAGGGA
1 *2B* (A)	CAAAG G.**TTTTC**CGG TAAGGGA
3 *2A* (D)	CAaAG G.**TTTTC**CGG TaAGGGA
*Pn*2	CAAAG G.**TTTTC**CGG TAAGGGA
*Pn*4	CRAAG G.**TTTTC**CGG TAARGGA
*Pn*1	CAAAG G.**TTTTC**CGG TAAGGGA
Pn NCTC 9336	CCAAA G**GTTTTC**CGG TAAGGGA
Pi ATCC 25611	CAAAG A**TTCAT**.CGG TGGAGGA
Pi ATCC 25611	CAAAG A**TTCAT**.CGG TGGAGGA

(b)

Pn ATCC 33563	C.AAA GGTTTT.CCG GTAAGGGA
Pn ATCC 25261	C.AAA GGTNTT.CCG GTAAGGGA
*Pn*5	C.AAA GGTTTTTCCG GTAAGGGA
*Pn*94	C.AAA GGTTTTYCCG GTAAGGGA
1 *2B* (A)	C.AAA GGTTTT.CCG GTAAGGGA
3 *2A* (D)	C.AAA GGTTTT.CCG GTaAGGGA
*Pn*2	C.AAA GGTTTT.CCG GTAAGGGA
*Pn*4	C.RAA GGTTTT.CCG GTAARGGA
*Pn*1	C.AAA GGTTTT.CCG GTAAGGGA
Pn NCTC 9336	CCAAA GGTTTT.CCG GTAAGGGA
Pi ATCC 25611	CCAAA GATTCA.TCG GTGGAGGA
Pi ATCC 25611	CCAAA GATTCA.TCG GTGGAGGA

KEY:

Pn ATCC 33563, *P. nigrescens* ATCC 33563; *Pn* ATCC 25261, *P. nigrescens* ATCC 25261; *Pn*5, *P. nigrescens* MH5; *Pn*94, *P. nigrescens* LM94; *Pn*2, *P. nigrescens* MH2; *Pn*4, *P. nigrescens* MH4; *Pn*1, *P. nigrescens* MH1; **1** *2B* (A), PLS isolate *P. nigrescens*; **2** *3A* (B), PLS isolate *P. intermedia;* **5** *1D* (C), PLS isolate *P. intermedia*; **3** *2A* (D), PLS isolate *P. nigrescens.*

Figure 4.3 Alignment of P-nig with PINLOs, *P. corporis* and *P. pallens*

Highlighted bases represent sequence identity between some other *Prevotella* species and primer P-nig.

```
      201                                                  250
  Pp1 .......... .......... AAAGATTTA. TCGGTAAAGG A.........
  Pp2 .......... .......... AAAGATTTA. TCGGTAAAGG A.........
  Pp3 .......... .......... AAAGATTTAT TCGGTAAASG A.........
 1156 .......... .......... AAAGATTTTA TCGGTATTGG A.........
 2160 .......... .......... AAAGATTTTA TCGGTATTGG A.........
   Pc .......... .......... AAAGA.TTCA TCGGTATGGG A.........
P-nig .......... .......... CAAAGGTTTT CCGGTAAGGG A.........
```

KEY:

*Pp*1, *P. pallens* strain 1037; *Pp*2, *P. pallens* strain 9423; *Pp*3, *P. pallens* strain 8792; 1156, PINLO HST 1156; 160, PINLO HST 2160; *Pc*, *P. corporis* ATCC 33547; P-nig, PCR primer P-nig.

Mättö *et al.* (1996b) reported cross hybridising strains when the *P. intermedia* probe 1Bi-1 was used which were identified on the basis of signal intensity and it was speculated that they may represent a subspecies of *P. intermedia*. Shah *et al.* (1995) also reported cross hybridization of 1Bi-1 at low stringency. This cannot be compared to the success as a PCR primer.

The specificity of the PCR reaction can be increased by minimising the incubation time between annealing temperature and extension temperature and thus reduce chances of mispriming (Saiki, 1989), although this is determined by the PCR machine. Reducing the primer and enzyme concentrations also limits mispriming (Saiki, 1989). Whilst this offers a path to help increase the specificity of P-nig, it cannot alter the lack of consistent priming of the intended target, which may require increased magnesium ions or a decreased annealing temperature (Roux, 1995).

In the event that species-specific amplification was seen or is seen on extension of this work, the primers should be tested for specificity against a large panel of species (Watanabe and Frommel, 1996). This could include the wide range of isolates identified from PLS patients (section 4.14) which includes *Leuconostoc* sp., *Neisseria* sp., *Peptostreptococcus* sp. and *Streptococcus* sp. In addition further *Prevotella* species must be included.

To summarise this section of work, the *P. intermedia* specific primers used here have potential for successful detection and identification of the species however further work is required to determine the specificity and sensitivity. The *P. nigrescens* primers proved highly inconsistent and require further optimisation before specificity and sensitivity issues can be addressed. Multiplex PCR results suggest that the combinations of P-int and 1Bi-1 plus RE-TPU1 are favourable but that P-nig and 2Bi-1 plus RE-TPU1 at the conditions tested are not.

4.6.8 EFFECT OF DNA EXTRACTION ON SUCCESS OF PCR

The effect of different DNA extraction methodologies to obtain a PCR template, has been discussed for RAPD-PCR (section 4.4.11) and PCR (section 4.6.8) and the techniques could be used to provide a template for PCR with specific primers. Agersborg *et al.* (1997) compared 3 techniques to cause cell lysis in *L. monocytogenes*; boiling cells, lysosyme and proteinase K and Triton X-100 detergent before PCR. With the exception of boiling cells to induce lysis, PCR amplification of extracted DNA was possible although in some cases the amount of product was minimal possibly due to the presence of inhibitory compounds

(Agersborg *et al.*,1997) suggesting that a more detailed extraction procedure is desirable. It was also reported that long term storage of proteinase K at -20°C has been shown to result in decreased lysis and ultimately poor amplification by PCR (Agersborg *et al.*,1997).

DISCUSSION

SECTION B: CLINICAL MICROBIOLOGY (PLS)

A multifaceted, progressive approach towards identifying oral bacterial isolates from patients with Papillon-Lefèvre Syndrome was undertaken.

It is important to note that the isolation of bacterial species from periodontal sites is dependant on many factors including the way in which samples are taken, the transport of samples, the isolation media and environment and the methods chosen for identification (see Slots, 1986b for review) and that the absence of an organism seen previously may reflect experimental conditions rather than a lack of association (Socransky *et al.*, 1987).

4.7 WHY IDENTIFY BACTERIA ISOLATED FROM CLINICAL SAMPLES ?

The identification of bacteria from clinical specimens is of interest to clinicians because it enables a specific diagnosis to be made and determination of appropriate antimicrobial therapy (Citron and Appelbaum, 1993). It also allows epidemiological information to be obtained (Citron and Appelbaum, 1993) which is of importance for transmission of pathogens and possible outbreaks of disease. However, it is difficult to attach significance to any one species that may be isolated from a mixed infection (Citron and Appelbaum, 1993) such as periodontal disease.

4.8 APPROACH TO IDENTIFICATION

As described in section 4.9 pure cultures with different colony morphologies that had been Gram stained were the starting point. Initially, the aim was to obtain a quick identification and overview of the organisms within the oral cavity by use of commercial identification kits. Therefore all pure cultures were tested in the identification kit appropriate to the Gram stain (sections 2.10.1 and 4.10.1.3) on the day they were received from MRI (section 4.9). It quickly became apparent that no identification would be reached for some isolates and that some further tests would be required both to identify some and clarify the identification of others. Thus the methodology adopted for identification evolved over time. Simple tests; catalase, oxidase, Gram stain were performed in all cases, oxidation-fermentation and growth in an alternative environment followed. Other tests were presumptive, adopted in the search for identifying certain species. This was particularly apparent were API suggested tests (requirement for V-factor, optochin resistance, nitrate reduction and EPS production) which could confirm the identification profile of a kit. GLC analysis was adopted at a late stage and as a final resort, partial 16S rDNA sequencing. As a result of this adaptive approach, not all isolates were tested in the same way for several reasons. Firstly, once the identification of an isolate was accepted it was not always available for

additional tests and secondly, although the majority of isolates were stored in FUM (appendix 6.1.5) at -80°C, some were unavoidably lost.

In the sections 4.9 - 4.11 following, the methodology as it was performed is discussed and in sections 4.12 - 4.14 the bacterial identifications are discussed. This is followed (section 4.15) by changes that could have been implemented.

4.9 OBTAINING PURE CULTURES OF ISOLATES

As described in section 2.9.2 gingival plaque samples were sent directly to a diagnostic microbiology laboratory at MRI routinely used by Turner Dental Hospital were they were incubated and pure cultures of aerobic and anaerobically growing isolates with different colony morphologies were obtained and all had been Gram stained. The MRI was used because of the quantity of samples, experience and thus the speed with which isolates could be purified. In addition, more than one person was assigned to the task. The initial aim of this section of work is described in section 4.8, to achieve this, the time in which pure cultures were available was considered important. Samples were sent on the day of sampling in Reduced Transport Fluid (RTF; see section 4.9.1) and plates of pure cultures were collected 5-10 days later. Anaerobically growing species were transported in an anaerobe jar (introduction section 1.8.1.3) to maintain the anaerobic environment.

4.9.1 REDUCED TRANSPORT FLUID (RTF)

Several transport media have been described and their effectiveness reported (Dahlén *et al.*, 1993; Deveaux *et al.*, 1990; Syed and Loesche, 1972). A transport medium must be able to preserve the reproducibility of bacterial cells whilst preventing their growth, either by a lack of nutritional substances or the inclusion of bacteriostatic substances (Deveaux *et al.*, 1990) and if to be used for anaerobes, protect from oxygen (Citron and Appelbaum, 1993).

RTF was chosen for use during this study and prepared by Dr. M. Abdi, University of Manchester, after being successfully used by a similar study at the Turner Dental School (Clerehugh *et al.*, 1996). This non-nutritional transport medium was first described for periodontal samples by Syed and Loesche (1972), although it does not contain bacteriostatic agents, the lack of nutrients prevent bacterial growth (Syed and Loesche, 1972). It contains dithiothreitol to lower the Eh (Deveaux *et al.*, 1990; Syed and Loesche, 1972) and EDTA to lessen bacterial aggregations (Syed and Loesche, 1972). One study (Deveaux *et al.*, 1990) comparing transport media, found that RTF displayed the best stability of bacterial counts when compared with glucose containing transport media and

tested with monoculture of *B. intermedius* (*P. intermedia* or *P nigrescens*) and paired culture of *B. intermedius* and *S. sanguis*. The use of RTF in preference to other transport mediums has been described (Deveaux *et al.*, 1990; Syed and Loesche, 1972), however the semisolid nutritious media VMGA (viability medium, Göteborg, anaerobically prepared and sterilised) III (Dahlén *et al.*, 1993) has been shown to be preferable for longer transport periods (Dahlén *et al.*, 1993).

4.9.2 SELECTIVE MEDIA

No selective media were used during this study. Partly this was due to the intended use of monoclonal antibodies to detect those species (*P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*) for which they may have been beneficial. Despite not using selective media, organisms including *Capnocytophaga* spp., pigmented *Prevotella* spp. and *Actinomyces* spp. were isolated, however, its use may have enabled isolation of other species associated with PLS (*E. corrodens*, *Fusobacterium* spp., *Haemophilus* spp., *Veillonella* spp.; Clerehugh *et al.*, 1996) or periodontal disease (*B. forsythus*; Darveau *et al.*, 1997). The review paper by Slots (1986b) details some selective media, others are given in the introduction section 1.8.1).

4.9.3 PURE CULTURE

The importance of a pure culture for identification purposes must not be underestimated. It can be defined as well isolated colonies that due to the method in which they were plated out they can be assumed to have developed from a single colony of the same organism (Cowan and Steel, 1993). If a culture is not pure when tested, abnormal test results will be seen. This may be either a mixture of characters from both organisms and no identification, or the mixture of characters may yield a totally different and false identification or there may be growth and identification of one species at the expense of the other (Cowan and Steel, 1993).

Plate cultures from MRI were assessed visually, accepted as pure and tested immediately. Culture information was not recorded, and the Gram stain morphology was accepted as supplied. Taking colony morphology into account and repeating the Gram stain would have improved the chances of selecting the correct identification kit and provided some information to add credence to the result. Little emphasis was placed on colony morphology, the colour was recorded were its was known to be important, in the case of black-pigmented bacteria. However, colony colour, size and shape and distinctive

morphologies such as pitting of the agar (aerobic culture, *E. corrodens*; anaerobic culture, *B. ureolyticus*); breadcrumb like colonies (*F. nucleatum*) or molar tooth colonies (*Actinomyces* spp.) can provide clues (Summanen *et al.*, 1993). A comprehensive list of descriptives commonly used to describe bacterial colonies plus diagrams can be found within the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993).

4.9.4 AEROBIC AND ANAEROBIC ISOLATES

Isolates were originally separated into aerobic and anaerobic growing isolates, this separation was the result of the initial isolation of pure cultures by staff at MRI. The labelling of isolates as either aerobic or anaerobic affected the way in which the isolates were tested and could potentially have biased the identifications expected. Organisms growing in an anaerobic environment were tested by GLC, however, GLC is a very valuable technique and all facultative anaerobes (which may have been in cultivated in an aerobic environment) should have been tested in this way.

This separation also meant that only isolates initially labelled as aerobic were tested in Hugh and Leifsons medium (oxidation-fermentation test); in reality valuable information would have been gained by testing any anaerobically growing isolates found to be positive for growth in an aerobic environment in this way. In part, these incomplete testing procedures were also due to the fact that the approach to identifications was adapted as required (see section 4.8).

The assumption of strict oxygen tolerances for all isolates is incorrect as a wide range of oxygen tolerances exist. It lead to some confusion with regards to the commercial identification kit in which isolates were tested. Kits marketed for anaerobes will identify microaerophilic or facultative organisms. The RapID ANA II system includes test results for *Actinomyces* sp., *Lactobacillus* sp., *S. constellatus*, *S. intermedius* and *G. morbillorum* (RapID ANA II system, instruction manual, 1995) and Rapid ID 32 A for *Actinomyces* sp. and *G. morbillorum* (Rapid ID 32 A, instruction manual, 1994). As a result many aerobically cultivated Gram-positive rods were tested in these kits to ensure these species were not overlooked. A further discussion on this topic can be found in section 4.10.1.3.

All processing of specimens was conducted at the bench, for aerobic cultures, this meant working in a short space of time to limit oxygen exposure before returning plates to the anaerobic cabinet (Summanen *et al.*, 1993).

No facilities were available to create a carbon-dioxide enriched atmosphere for those species preferring enhanced CO_2 such as *Haemophilus*, *Eikenella*, *Capnocytophaga* or

microaerophilic streptococci (Summanen *et al.*, 1993). However, despite this capnophilic and microaerophilic species were isolated from aerobic (*S. milleri* group) and anaerobic culture (*S. milleri* group and *Capnocytophaga* spp.). *Haemophilus* spp. and *Eikenella* spp. have been isolated from PLS patients in the past (Clerehugh *et al.*, 1996) but were not seen during this study.

4.10 COMMERCIAL IDENTIFICATION KITS USED FOR THE IDENTIFICATION OF MICROORGANISMS FROM PATIENTS WITH PLS

4.10.1 COMMERCIAL IDENTIFICATION KITS

Many commercial identification kits are available for the quick and simple identification of medical bacterial isolates (Busse *et al.*, 1996). The initial aim of the study was to obtain a quick identification and therefore overview of the organisms residing within the oral cavity, to this end, all isolates were put through a commercial identification kit as soon as received in pure culture from MRI.

4.10.1.1 Use of identification kits

Identification kits are quick and easy to use (Busse *et al.*, 1996) with identifications typically obtained within 4 h, however added to this is at least 24 h required to culture the organism to provide an inoculum of the correct turbidity (Pattyn *et al.*, 1993). They provide a simple alternative to performing 20-30 characterisation tests individually, although the user may choose to supplement the results with additional tests (Summanen *et al.*, 1993). A miniaturised standard procedure has been described for the identification of anaerobic bacteria (Pattyn *et al.*, 1993) that incorporates, 11 biochemical tests and GLC analysis. Whilst it took longer to complete than the Rapid ID 32 A kit, it correctly identified 100% of isolates tested to genus level and 94.7% to species level. In addition it was estimated to be 25 times cheaper (after GLC investment) than Rapid ID 32 A (Pattyn *et al.*, 1993). The usefulness of a miniaturised standard procedure to identify anaerobes is dependant on GLC equipment and is therefore not applicable to all laboratories. It does however illustrate the fact that commercial identification kits are not necessary for successful identification but are a valid resource.

The identification reached is limited by the numbers of strains which are characterised and included in a database, against which all comparisons are made (Busse *et al.*, 1996), the result is that strains are assigned identifications based on organisms they closely resemble (Adney and Jones, 1985) which may not always be correct. New species will be missed by

commercial kits (Busse *et al.*, 1996) as the new species names are not included within the database (an example of this is *P. pallens*, see section 4.18) but a kit will highlight atypical species which can be tested further. Reading the results is dependant on the correct interpretation of a colour change reaction that is not always easy to visualise (Summanen *et al.*, 1993), especially using the RapID ANA II system which requires user interpretation of a distinct colour change (dark pink) compared to a pale one (pale pink; Adney and Jones, 1985; Appelbaum *et al.*, 1985). Commercial kits will not always provide a species level identification, for example, the RapID ANA II system will not give species level identifications for *Capnocytophaga*, *Bifidobacterium*, *Mobiluncus* or *Veillonella* species (Celig and Schreckenberger, 1991) and Rapid ID 32A will not type *Mobiluncus* or *Veillonella* species (Pattyn *et al.*, 1993). They can also fail to differentiate closely related species e.g. *P. intermedia* and *P. nigrescens* (personal experience, see section 4.17.1.1) *and P. corporis* (Moll *et al.*, 1996). They can only be used when it is known which genera are expected, in other words Gram morphology has been determined (Busse *et al.*, 1996). Limitations including effect of altered inoculum size and incubation time must be remembered as well as the potential for altered reaction profiles between fresh isolates and subcultured strains (Busse *et al.*, 1996).

4.10.1.2 Success of identification kits

Only 22 out of 53 (41.5%) isolates labelled as aerobic and facultative and 12 out of 32 (37.5%) of isolates labelled as anaerobic and facultative were identified by commercial identification kits. Of these positive identifications, the majority of aerobes (14 out of 22) were identified using Rapid ID 32 Strep which is a reflection of the greater number of streptococcal species isolated compared to other aerobes and facultative species. The majority of anaerobes (8 out of 22) were identified with Rapid ID 32 A, but three isolates achieved the same identifications with this kit and the RapID ANA II system.

The success of commercial identification kits cannot be directly compared unless it is clear that an isolate was tested with the correct kit (see section 4.10.1.3) however positive identifications were achieved using Rapid ID 32 Strep, Rapid ID 32, API NH and RapID ANA II system. The lack of positive identifications with API 20 NE and API Coryne is more likely due to the incorrect choice of kit rather than the ineffectiveness of these kits.

The limitations of commercial identification kits are recorded in the literature, Appelbaum *et al.* (1985) described variation in accuracy and reproducibility of the RapID ANA system (fore-runner to Rapid ANA II) between laboratories, with inoculum size and

homogenisation of the inoculum suspension particularly important (Barbé *et al.*, 1994). The problem of reproducibility is inferred by comparing the later studies of Celig and Schreckenberger (1991) and Marler *et al.* (1991) who investigated the performance of the RapID ANA II system. Celig and Schreckenberger (1991) reported 94% of the 300 isolates tested were identified to genus level and 87% to species level, whilst Marler *et al.* (1991) reported 68% of the 566 isolates were identified to species level. The greatest discrepancy between the two reports is in the identification of anaerobic Gram-negative bacilli with Celig and Schreckenberger (1991) quoting 86% correct identification to species level whilst Marler *et al.* (1991) quoted only 62%. It was concluded that whilst RapID ANA II system was effective at identifying the majority of isolates, the use of additional tests especially GLC would enhance its effectiveness, especially for Gram-positive rod-shaped isolates (Marler *et al.*, 1991), including *Actinomyces* species (Miller *et al.*, 1995), the identification of which is problematical (Moll *et al.*, 1996). This is illustrated by this study, where the use of simple tests and GLC aided and improved identification of all isolates, a principle that was applied to all commercial identification kits used during this study.

Interpretation of the microcode produced from the RapID ANA II system relies on knowledge of the Gram morphology and searching for the microcode within the correct group; (i) Gram-negative anaerobic bacilli, (ii) Gram-positive anaerobic bacilli and (iii) anaerobic cocci (Celig and Schreckenberger, 1991), highlighting the importance of performing some basic microbiological procedures before testing with commercial kits. Rapid ID 32 A does not take the Gram morphology into account which can lead to misidentifications (Pattyn *et al.*, 1993); another argument for supplementing results of identification kits with other tests. However a success rate of 96.7% correct identification to species level (Pattyn *et al.*, 1993) has been reported for the Rapid ID 32 A kit. A 73.8% species level identification of *Neisseria* and 90.3% of *Haemophilus* sp. has been reported by the API NH kit (Barbé *et al.*, 1994). The API NH kit has been shown readily to differentiate *Neisseria gonorrhoeae* and *N. meningitidis* but *N. cinerea* is sometimes confused with *N. gonorrhoeae* when further tests are required for identification (Barbé *et al.*, 1994). *N. flavescens* and *N. denitrificans* are not included within the API NH database (Barbé *et al.*, 1994), despite reported similarities to *N. subflava* (Barrett and Sneath, 1994), *N. flavescens* is often identified by API NH as *N. gonorrhoeae* or *N. cinerea* (Barbé *et al.*, 1994) as was the case here. In this study the identification was suggested by rRNA sequence analysis, however pigment production by *N. flavescens* will distinguish strains from other *Neisseria* including *N. gonorrhoeae* and *N. cinerea* but excluding *N. subflava*

(Cowan and Steel, 1993). The importance of additional tests to supplement database information and obtain identifications is also recorded for API Coryne (Funke *et al.*, 1997), it is hypothesised that this is a consequence of constantly changing taxonomy (Funke *et al.*, 1997).

Bascomb and Manafi (1998) tested numerous identification kits for the successful identification of Gram-positive cocci, including Rapid ID 32 Strep. The Rapid ID 32 Strep displayed the highest possible number of taxa identified (52) compared for example with the RapID STR (Innovative Diagnostics, Atlanta, GA) which identifies 26 taxa. The biggest drawback of this kit was described as the requirement for a heavy inoculum (4.0 MacFarland Scale), compared to 1.0 (MacFarland Scale) for the RapID STR and thus the need for overnight growth before testing (Bascomb and Manafi, 1998). A success rate of 70.2-82.2% has been reported for the Rapid ID 32 Strep kit (Bascomb and Manafi, 1998). The reported success rates of identification kits are much higher than seen during this study. Despite the low numbers of isolates, the percentage correctly identified by API NH, Rapid ID 32 A and the RapID ANA II system does not reach 50%. Only the Rapid ID 32 Strep kit identified over 50% (60.8%) correctly, but still does not reach reported successes (Bascomb and Manafi, 1998). In the light of these facts, it is probable that misidentifications during this study are more likely to be due to an incorrect choice of identification kit or species not represented within the code database, rather than poor performance of a particular kit.

4.10.1.3 Choice of identification kit

The isolates were tested in an identification kit deemed applicable to the Gram morphology and relying on the Gram stain as performed by staff at MRI and whether or not they were described as aerobic or anaerobic isolates. Anaerobic organisms were tested with either the Rapid ID 32 A kit (Gram-negative organisms) or Rapid ANA II system (Gram-positive organisms). Both anaerobe kits will detect microaerophiles and capnophiles (see section 4.9.4) and for this reason any aerobic isolates which did not obviously fit into another category were tested by one of these kits. Gram-negative aerobes (*Neisseria*, *Haemophilus* and *Branhamella*) were tested in API NH, API 20 NE was also used for Gram-negative rods. Aerobic Gram-positive bacilli should have been tested with the API Coryne kit, to test for *Actinomyces*, *Propionibacterium* and *Rothia* (Funke *et al.*, 1997), in reality most were tested with Rapid ANA II (see section 4.9.4). Gram-positive cocci were tested with the Rapid 32 Strep kit, ideally all Gram-positive cocci should have been tested for catalase

production first and any catalase-positive tested with the Rapid ID 32 Staph kit. The oral streptococci are facultative anaerobes (Hardie and Whiley, 1992) and any Gram-positive cocci incubated anaerobically would have benefited from testing with this kit, although the RapID ANA II system does detect several streptococci species (section 4.9.4).

It should be noted that other identification kits are available (see Busse *et al.*, 1996 for comprehensive list) and those used here were the ones regularly used in our laboratory.

4.10.1.4 Problems with commercial identification kits as used here

It is clear that the choice of identification kit without some preliminary tests is not without problems. Without an accurate Gram morphology or determining oxygen tolerance correctly, facultative species labelled as aerobic (especially Gram-positive bacilli) or labelled as anaerobic (Gram-positive cocci) would not be tested in the correct kit. The use of an incorrect kit and possible use of a second kit is a waste of time, money and resources and may lead to more expensive and lengthy techniques (such as partial 16S rRNA gene sequencing) being adopted unnecessarily.

4.10.1.4.1 Isolates labelled as aerobic and facultative

Fourteen (**1** *1A*, **1** *2B*, **2** *1A*, **2** *1B*, **3** *1B*, **3** *1E*, **3** *2A*, **5** *1A*, 7 *2F*, **8** *1A*, **9** *2E*, **9** *2F*, **10** *1A* and **11** *1B*) aerobic isolates were tested with either Rapid ID 32 A or Rapid ANA II system. Of these the identifications of 4 (**2** *1A*, **2** *1B*, **8** *1A* and **10** *1A*) were accepted, three (**1** *2B*, **3** *1E* and **3** *2A*) were put into an anaerobe kit as a result of operational error. Three (**1** *1A*, **3** *1B* and **11** *1B*) were tested in both API Coryne and an anaerobe kit to cover anaerobic and facultative metabolism and the remaining 4 isolates (**5** *1A*, 7 *2F*, **9** *2E* and **9** *2F*) plus 1 tested in error (**1** *2B*) would have benefited from testing by both types of kit. Of the 10 isolates not identified by anaerobe identification kit, 4 (**1** *2B*, **3** *1E*, **3** *2A* and **9** *2E*) were identified by another method and 6 (**1** *1A*, **3** *1B*, **5** *1A*, 7 *2F*, **9** *2F* and **11** *1B*) were not identified, 4 of which was due to loss of viability.

Isolate **3** *2A* was tested in an inappropriate kit and should have been tested with API NH, similarly isolates **5** *2A* and **10** *2A*, due to misleading Gram stain information, should have been tested using Rapid ID 32 Strep.

4.10.1.4.2 Isolates labelled as anaerobic and facultative

As explained above, facultative streptococcal species labelled as anaerobic could have been identified using Rapid ID 32 Strep, only 1 Gram-positive coccus (**2** *2A*) was tested this way, when a further 8 isolates (**2** *2B*, **3** *1D*, **6** *1A*, **11** *1A*, **12** *2A*, **13** *2A*, **18** *1D* and **18** *2A*) could have been. The use of partial 16S rRNA gene sequencing and GLC analysis assigned 3 (**2** *2B*, **6** *1A* and **12** *2A*) of the 8 to *Streptococcus*. With the exception of isolate **18** *1D*, the other Gram-positive cocci were identified as *Peptostreptococcus* species. In the absence of testing for oxygen sensitivity first, two kits would have been required to ensure *Peptostreptococcus* was not missed, but it would have ruled out expensive and time consuming sequence analysis.

4.11 SUBSEQUENT IDENTIFICATION

As described in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (1993), the use of character tests for identification depends on choosing those which are constant and reproducible and comparing the characters of the organism in question to characters of known organisms. In many cases, a genus level identification can be achieved quite easily using a few tests detailed in the first and second stage identification tables of Cowan and Steel (1993). The results of catalase and oxidase tests, carbohydrate breakdown and determination of oxygen tolerance were compared, for the majority of isolates, to commercial kits with additional tests chosen as appropriate such as spore stain and nitrate reduction which were not performed in all cases and considered unnecessary for identification of all isolates.

In many cases, a positive and negative result are easy to distinguish, however control organisms should be considered if they are available. These are organisms which have known reactions to the test under consideration and can aid interpretation and ensure that the test is being performed correctly (Cowan and Steel, 1993).

4.11.1 GRAM STAIN AND CELLULAR MORPHOLOGY

The Gram stain is the starting point for bacterial species identifications and must be performed and interpreted with care.

There are potential problems that must be considered when performing and observing Gram stains. It is vital to test a young culture because a Gram-positive organism becomes Gram-negative with age and the solvents (acetone) used to decolourise cells, in superfluous amounts will remove the crystal violet-iodine complex from cells of all ages and should

therefore be rinsed off thoroughly and quickly. In addition, the apparent variable staining nature of some groups of bacteria (*Gemella* and *Mobiluncus*) should not be forgotten (see introduction section 1.8.2.1). For this reason, the identification of isolate **2** *1B* (two isolates, one aerobic and one anaerobic) as *Mobiluncus* spp. was not discounted despite their Gram-positivity.

Light microscopy of a Gram stained preparation provides information concerning cell morphology, it is not always possible to determine coccal or rod shape of cells and the term coccobacilli is used. Cell morphology is affected by the growth conditions; medium and temperature, cultures grown in liquid medium are suggested for Gram stain (Holdeman *et al.*, 1977). The *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977) displays the morphology of anaerobic species grown in two types of broth; peptone yeast extract with and without glucose and is a useful aid to interpret Gram stains of anaerobic organisms. Morphological type is especially useful when studying Gram-positive cocci (Facklam and Elliott, 1995) as species of *Enterococcus*, *Lactococcus* and *Streptococcus* will form chains, *Leuconostoc* occurs in pairs (Cowan and Steel, 1993) and chains (Cowan and Steel, 1993; Facklam and Elliott, 1995), *Gemella* forms tetrads (Bascomb and Manafi, 1998) whilst cells of *Staphylococcus*, *Micrococcus* and *Stomatococcus* form clusters (Facklam and Elliott, 1995).

The importance of ascertaining correct Gram morphology before using a commercial kit is discussed in section 4.10.1.3. In the case of 1 aerobic isolate **9** *2E*, which was identified as a Gram positive rod and therefore tested with RapID ANA II as described in section 4.9.4, the Gram-morphology does not appear to fit with the final identification achieved. Isolate **9** *2E* was identified as the Gram-positive coccus *Leuconostoc mesenteroides* by partial 16S rRNA sequencing with an identity level of 99.3% over 458 bp. This organism occurs in pairs or short chains (Cowan and Steel, 1993) and it is possible that the occurrence of pairs of cells were mistaken for rods, it has also been described as a coccobacilli (Bascomb and Manafi, 1998) and therefore the identification was accepted. Isolate **3** *2A* (aerobic) should have been tested by API NH (section 4.10.1.3) however, due to testing with the incorrect kit, this isolate was studied by sequence analysis. A suggested identification of *N. elongata* was obtained, which did not appear to fit with the Gram morphology. This example illustrates the importance of considering the species level identification as well as the genus, because whilst *Neisseria* are Gram-negative cocci, bacillary forms of *N. elongata* have been reported (Cowan and Steel, 1993). The Gram morphology of isolate 7 *2B* (anaerobic) as a Gram-positive bacilli appears to rule out the suggested identification of *S. gordonii.*

However, streptococci can appear as short rods (Tanner *et al.*, 1994) and the identification was accepted.

The majority of Gram stains yielded results correlating with those obtained at MRI, with the exception of 2 aerobically cultivated and 3 anaerobically cultivated isolates. Two aerobically cultivated isolates **5** *2A* and **10** *2A* were reported as Gram-negative cocci and therefore tested in API NH, later Gram stains revealed Gram-positive cocci which was more plausible in view of other test results and sequence analysis. There are several possible explanations. Firstly the original Gram stain was correct and the second due to contamination, however poor results were obtained with API NH, so either the contamination was interfering with the test result or the isolates were in the wrong kit. In which case, the Gram morphology was probably wrong and as Gram-positive cocci the Rapid ID 32 Strep kit would have been chosen (unless catalase-positive). The most likely explanation in this case is that the original Gram stain was taken from an older culture which had lost its Gram-positivity, thus illustrating how selecting a culture of the correct age can affect the identification procedure. The three anaerobic isolates with differing Gram strain results are not so simple to explain. Isolate **1** *2B* was finally identified as *P. nigrescens* (isolate A, section 4.17) a Gram-negative rod, despite an original Gram-positive result whilst the other two isolates **10** *2B* and **13** *2A* were unidentified. A rod, isolate **10** *2B* was first recorded Gram-negative then Gram-positive and it is primarily the cell shape which discounts the Rapid ID 32 A identification of *Pstr. micros*. That is until the other test results are regarded, as this isolate is catalase negative and grows aerobically. The results for isolate **13** *2A* were seen in the same way except the cells were coccoid. The original identification of *Pstr. anaerobius* tallied with the first Gram stain, however this isolate grew aerobically. It is possible as with **5** *2A* and **10** *2A* above, older cultures were stained and therefore seen as Gram-negative instead of Gram-positive, but this does not aid the identification based on the results obtained. If this had been the case maybe **13** *2A* should have been tested in the Rapid ID 32 Strep kit or perhaps the only answer would have been sequence analysis.

4.11.2 AEROBIC AND ANAEROBIC CULTURE

Initial plating of a specimen must be done as soon as is possible after sample taking. Once plates are incubated they should not be removed from the anaerobic environment before 48 h. A plate culture should be incubated for seven days before being called negative

(Summanen *et al.*, 1993). As described in section 4.9.4, artificial separations into aerobic and anaerobic isolates occurred. Testing whether isolates grew in an alternative environment added the term facultative to the identifications. Tables 3.7-3.9 list final identifications of those isolates originally labelled as aerobic, whilst tables 3.16 and 3.17 list those originally labelled as anaerobic. From this point, isolates are categorised as obligate aerobes or anaerobes, facultative anaerobes or capnophiles which provides a truer representation of the range of oxygen tolerance displayed by bacteria. None are labelled as microaerophilic (aerobes requiring reduced concentrations of oxygen, although *Actinomyces* spp. and '*S. milleri*' group organisms could have been labelled as such.

There are a couple of identifications called into question by the accepted oxygen tolerance and that recorded here. Isolate **3** *1E* (aerobic) identified as having the closest phylogenetic relationship to *Kingella indologenes*, now *Suttonella indologenes* (Dewhirst *et al.*, 1990) was recorded as able to grow in an anaerobic environment, however whilst the genus *Kingella* are known to be facultatively anaerobic (Cowan and Steel, 1993), *S. indologenes* is reportedly aerobic (Dewhirst *et al.*, 1990) with growth enhanced by CO_2. This correlates with the observation of growth made here and the identification and grouping remains unchanged. Isolate **2** *1B* (aerobic) was identified as *Mobiluncus* spp. an anaerobic bacterium (Spiegel and Roberts, 1984) which was shown to be able to grow in an anaerobic environment but was cultured predominantly in an aerobic environment suggesting a facultative nature. Isolate 7 *2B* (anaerobic) was found to be most similar to *S. gordonii* by sequence analysis, however it did not grow in an aerobic environment whereas streptococcal species are recognised as facultative anaerobes (Cowan and Steel, 1993). *G. morbillorum* (**18** *1D*, anaerobic) requires anaerobic conditions on cultivation, but becomes aerotolerant during laboratory culture (Facklam and Elliott, 1995).

4.11.3 CATALASE TEST

The catalase test is traditionally used to divide the catalase-positive *Micrococcaceae* (which includes the genera *Micrococcus*, *Staphylococcus* and *Stomatococcus*) and the catalase-negative *Streptococaceae* (which includes *Streptococcus, Leuconostoc, Gemella* and *Lactococcus*) families (Bascomb and Manafi, 1998). This is complicated by the catalase-variable activity of *Stomatococcus*. A method other than the one described here (section 2.10.2.2) can be used to detect catalase activity (Cowan and Steel, 1993; Summanen *et al.*, 1993). H_2O_2 can be dropped directly onto a colony on a plate and viewed for effervescence, however, colonies grown on blood agar are unsuitable for this because the

haemoglobin in red blood cells can breakdown H_2O_2 which causes false positive results. Colonies tested during this study were grown on blood-FAA before testing for catalase activity, to compensate for this, care was taken to avoid touching the agar whilst removing a colony for test (Summanen *et al.*, 1993). During this study 3% H_2O_2 was used, however it has been reported that 15% gives better results when testing anaerobic bacteria (Summanen *et al.*, 1993).

Positive controls for this test are *Staphylococcus aureus* (Cowan and Steel, 1993) and *S. epidermidis* (Collins *et al.*, 1995) and negative ones are *Streptococcus pyogenes* (Cowan and Steel, 1993) and *E. faecalis* (Collins *et al.*, 1995).

4.11.4 OXIDASE TEST

An alternative method for performing an oxidase test, other that that used here (section 2.10.2.3) has been described (Cowan and Steel, 1993) to test broth or plate cultures directly. It is less sensitive and requires two solutions instead of one but prevents false positives caused by nichrome or dirty wire loops (Collins *et al.*, 1995). The positive purple colour is quite distinctive and it is seldom difficult to interpret the result, however only colour changes occurring within 30 s should be accepted. Positive and negative control organisms for this test are *P. aeruginosa* and *A. lwoffii* respectively (Collins *et al.*, 1995; Cowan and Steel, 1993), *E. coli* has also been recommended as a negative control (Cowan and Steel, 1993).

4.11.5 OXIDATION-FERMENTATION TEST

The oxidation-fermentation test is described in section 1.8.2.3.3 of the introduction. This test determines whether an organism undergoes oxidative or fermentative metabolism (Hugh and Leifson, 1953). It is described here using the indicator bromothymol blue, the use of bromocresol purple has also been described (Collins *et al.*, 1995). Bromothymol blue turns from blue to yellow as acid lowers the pH (Collins *et al.*, 1995). Bromocresol purple can also be used (Collins *et al.*, 1995), the difference being the lower pH range (yellow at pH 5.8 and blue at pH 6.8; Collins *et al.*, 1995). Paraffin wax is traditionally used to create anaerobic conditions although melted Vaseline or agar can also be used (Collins *et al.*, 1995). Alternatively, one tube can be incubated in an anaerobic environment (Collins *et al.*, 1995).

The formation of acid due to the breakdown of the carbohydrate causes the indicator to change colour and the metabolism to be determined (Hugh and Leifson, 1953). The colour

change is clear and there is little problem determining the results, however control organisms can be used. Collins *et al.* (1995) suggest *Acinetobacter calcoaceticus* for oxidation, *E. coli* for fermentation and *Alcaligenes faecalis* as a test for no action whilst Cowan and Steel (1993) suggest *P. aeruginosa* (oxidation), *Serratia marcescens* (fermentation) and *Actinetobacter lwoffi* (no action).

4.11.6 SPORE FORMATION

Spores are produced by the Gram-positive genera *Clostridium* and *Bacillus* in response to nutrient depletion. They consist of a core surrounded by an inner germ cell membrane, a thick cortex and a spore coat which ensure a resistance to heat, drying, chemicals and radiation allowing spores to survive and germinate under more favourable conditions. As well as the ability to form spores, the shape and location of within cells can provide species information, for example the spores of *Clostridium* species are oval or spherical and can cause the bacterial cell to swell, although, spores are frequently observed outside of the cells in stained preparations (Cowan and Steel, 1993). Occasionally, organisms may lose the ability to form spores, either due to the medium used, temperature or a deficiency of manganese in the medium (Cowan and Steel, 1993).

There are two methodologies described in Cowan and Steel (1993) for performing a spore stain, both relying on the action of heat to force dye into the spore and staining it. The dye is removed only from the vegetative cell by water, allowing them to take up the counterstain. In this study spores were stained green with malachite green and vegetative cells red with safranin. In an alternative method (Cowan and Steel, 1993) spores were stained red by carbol fuchsin and cells blue with Loeffler's methylene blue.

As an alternative to staining, spores can be viewed by phase contrast microscopy (Cowan and Steel, 1993) or their presence confirmed by showing that the culture can survive heating at 80°C for 10 minutes (Cowan and Steel, 1993).

4.11.7 NITRATE REDUCTION

The ability to reduce nitrates is a characteristic of species including members of the genera *Actinomyces*, *Neisseria* and *Kingella* making it a valuable test for species identification in some cases. *Kingella denitrificans* reduces nitrate whilst other members of the genus do not (Morse and Knapp, 1992). *N. mucosa* is the only member of *Neisseria* which reduces nitrate (Morse and Knapp, 1992) and of the Actinomycetes, *A. naeslundii* and *A. odontolyticus* have this characteristic (Schaal, 1992). Only suspected *Neisseria* spp. were

tested for ability to reduce nitrates when it would have been useful to confirm the identity of isolate **16** *1A* (aerobic) as *K. denitrificans* and rule out the identification of either *A. naeslundii* or *A. odontolyticus* for isolate **10** *1B* (anaerobic).

Cowan and Steel (1993) describe 5 methods to test for nitrate reduction based on addition of reagents and a colour change which indicates the presence of nitrites indicating the reduction of nitrates.

A positive control for nitrate reduction is *S. marcescens* and a negative one *A. lwoffii* (Collins *et al.*, 1995; Cowan and Steel, 1993).

4.11.8 EXTRACELLULAR POLYSACCHARIDE (EPS) PRODUCTION

The production of EPS (dextran or levan) is a function of some oral streptococcal species. EPS producers include *S. mutans*, *S. oralis*, *S. sanguis* (dextran) and *S. salivarius* (levan), whilst strains of the *S. milleri* (*S. anginosus*, *S. constellatus*, *S. intermedius*) group (Collins *et al.*, 1995) and *S. mutans* (Hardie and Whiley, 1992) do not produce it. The characteristic was used here to confirm the identities of two isolates **3** *1C* (aerobic) as *S. sanguis* and **9** *1B* (aerobic) as *S. oralis*. Twenty-four isolates were ultimately identified as *Streptococcus* spp., but only 7 were tested for the ability to produce EPS. During this study colony growth on TYC was used to test EPS production but it can also be tested for in broth with an in increase in viscosity associated with its production (Cowan and Steel, 1993).

EPS production can be used to provide a presumptive identification (Hardie and Whiley, 1992) due to the distinctive colony morphology on TYC agar, unfortunately this characteristic was not fully exploited here. The use of EPS production should always be paralleled with other tests as it is a variable characteristic of some (*S. gordonii*, *S. oralis*, *S. sanguis*) species (Hardie and Whiley, 1992). Some typical colony morphologies of EPS producers on TYC are provided below (Hardie and Whiley, 1992):

- *S. gordonii*: similar to *S. sanguis*. Additional techniques are required to distinguish *S. sanguis* and *S. gordonii*.
- *S. mutans*: displays grey, white or yellow irregular shaped colonies with a rough surface, a heaped appearance and a crumbly texture. Colonies appear to be in the centre of a pool of EPS.
- *S. oralis*: displays two colony morphologies, one similar to *S. sanguis* but of a smaller diameter, and one smooth and non-adherent. Additional techniques are required to distinguish *S. sanguis* and *S. oralis*.

- *S. salivarius*: displays white or grey, smooth doomed colonies which are large and mucoid.
- *S. sanguis*: colonies are colourless, white or grey with either a smooth or rough appearance, they may be difficult to remove from the agar surface.

In addition to commercial identification kits, identification tables based on many properties are available for *Streptococcus* spp. (Cowan and Steel, 1993; Hardie and Whiley, 1992). In addition Kikuchi *et al.*, (1995) describe the identification of viridans streptococci and Facklam and Elliott (1995) describe those procedures required to differentiate Gram-positive, catalase-negative cocci that do not belong to *Streptococcus* or *Enterococcus*.

4.11.9 V-FACTOR

V-factor (nicotinamide adenine dinucleotide NAD) is a coenzyme required by *Haemophilus* spp. A requirement for it can be determined by satellitism around a colony of *Staphylococcus aureus* and it is a useful way of identifying suspected *Haemophilus*. *H. parainfluenzae* can be used as controls to demonstrate satellitism (Cowan and Steel, 1993). *Haemophilus* spp. also require X-factor (haemin) which occurs in trace amounts in most media (Cowan and Steel 1993), *H. influenzae* requires both X- and V-factors and can therefore be used as a control organism (Cowan and Steel 1993). If the test for V-factor had resulted in *Haemophilus* spp. they would have been cultivated on chocolate agar which is blood agar heated to release V- and X-factors (Cowan and Steel, 1993).

4.11.10 GLC

The theory and details of GLC are covered in section 1.8.4. It is used to analyse the products of fermentation which vary between groups of bacteria according to the nature of the fermentation and therefore indicative of a bacterial genus (Summanen *et al.*, 1993). Used in conjunction with other tests, GLC analysis is useful for identification (Pattyn *et al.*, 1993; Summanen *et al.*, 1993). Qualitative analysis is sufficient for analysis (Henis *et al.*, 1966; Holdeman *et al.*, 1977) with the occurrence of a particular acid or alcohol recorded by comparison of retention times of standards (tabulated in section 6.19; tables 6.19 and 6.20). The *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977) suggests the use of 10 volatile acid standards (formic, acetic, propionic, *iso*-butyric, butyric, *iso*-valeric, valeric, *iso*-caproic, caproic and heptanoic), 6 alcohol standards (ethanol, propanol, *iso*-butanol,

butanol, *iso*-pentanol and pentanol) and 8 non-volatile acid standards (pyruvic, lactic, oxalacetic, oxalic, methyl malonic, malonic, fumaric and succinic). Note that formic acid is not detected by FIDs (section 1.8.4.6). In this study only the volatile acids acetic, propionic, *iso*-butyric, butyric, *iso*-valeric and valeric; alcohol's ethanol, propanol and butanol and non-volatile acids lactic, oxalic, malonic and succinic were used. These were chosen because they were readily available. The lack of volatile standards was compensated for by the knowledge of the linear relationship (separate for straight and branched chain acids) under isothermal conditions between the number of carbon atoms and log of the retention time (Drucker, 1981) allowing unknown peaks to be named. The last peak seen during analysis of isolate **11** *1A* (anaerobic) was classified as *iso*-caproic acid this way. Unfortunately the equivalent relationship for non-volatile acids is not as simple and works only of each class of compound, lactic acid is a hydroxy-acid and succinic is a dicarboxylic acid (Drucker, 1981). The order of elution of compounds is constant and thus helps name uncertain peaks. The sample can be mixed with a standard to obtain confirmation of the peak, if it is the same substance the peak will appear larger than either sample or standard alone and there will be no additional peaks (Drucker, 1981). This was found to be particularly important were slight drifts in retention times were seen due to continuous operation (Henis *et al.*, 1966) or a substance for which there was no standard was eluted such as the production of fumaric acid by isolate **10** *1A* (anaerobic). Despite these methods to compensate for a lack of standards there was one peak with a retention time after succinic acid, seen in analysis of isolate **11** *1A* (anaerobic) that was unidentified. It is possible that it represented carry over of sample between programme runs, although standardisation of equilibration time and occasional reconditioning of the column should have prevented this. The GLC profiles obtained for two isolates were very different from those recorded by Summanen *et al.* (1993). *Pstr. micros* has a major acetic acid peak and *Pstr. anaerobius* has acetic, iso-valeric, iso-butyric, iso-caproic and butyric acid peaks (Summanen *et al.*, 1993). The profiles of neither isolate (**3** *1D* and **18** *2A*) showed similar results but the identifications were accepted. These typical results are from organisms grown in peptone-yeast-glucose broth which may account for the differences seen here, as iso-caproic acid is reported to be a reliable identification characteristic for *Pstr. anaerobius* (Murdoch and Mitchelmore, 1991). Alternatively there was not enough growth to obtain a satisfactory result. A lack of volatile fatty acid production has been reported for *Pstr. micros* (Murdoch and Mitchelmore, 1991).

Both the *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977) and the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) detail protocols suitable for the detection of anaerobes, however as described in the introduction (section 1.8.4.2) parameters will be affected by the column packing, the sample and the type of detector. Microbiological Applications of Gas Chromatography (Drucker, 1981) contains theory and advice regarding GLC set-up and operation. The column packing (6% Carbowax 20M on Chromosorb W AWDMCS - see abbreviation list) used in this study was selected as one previously used successfully by Jenkins (1988) during a study of the taxonomy of *B. fragilis* and related species. The column packing; Chromosorb, is a porous polymer suitable for the analysis of acids and alcohols (Drucker, 1981). Carbowax is the stationary phase which coats the Chromosorb; it has a temperature range of 60-220°C. The volatile acid and alcohols standards were used to define an optimal temperature profile combined with knowledge and experience of suitable temperature ranges (Drucker, personal communication). An isothermal temperature profile failed to resolve ethanol, therefore, the initial column temperature was adjusted to increase from 65°C to 115°C to ensure separation of peaks produced by ether and ethanol.

Qualitative GLC analysis was undertaken for the majority of anaerobically growing isolates, but as mentioned in section 4.9.4, it could have been used for the facultative species which were growing in a predominantly aerobic environment. Table 6.21 in appendix 6.20 lists end-products representative of genera. The most commonly eluted volatile acid was acetic with lactic and succinic the most common non-volatile acids. The end-products were labelled as major and minor peaks (see figures 3.17-3.27) according to convention (Holdeman *et al.*, 1977). However, it must be noted that in this case the terms major and minor are relative and infer to the isolate under test. In most cases if only one peak was seen, it was termed major regardless of size. No quantitative analysis was performed thus peak sizes cannot be compared across samples.

In the case of one isolate (**2** *1B*; anaerobic) which was identified as *Mobiluncus* spp., the GLC profile did not contain acetic acid, the major product of this species. It is unknown whether this indicates an incorrect identification, an atypical strain, or is a function of growth conditions. *Eubacterium* spp. produce an array of different profiles (Holdeman *et al.*, 1977) but most produce lactic acid and Summanen *et al.* (1993) report there may be no major acids leading to acceptance of the identification of isolate **1** *2A* (anaerobic). A review of the genus *Eubacterium* by Andreesen (1992) tabulates fermentation end-products of the

different *Eubacterium* species. Of those associated with the oral cavity, *E. brachy* and *E. timidum* display variable production of formate (which would not be detected) and lactate which could result in no major peaks.

In 4 cases, the formation of methyl-esters of non-volatile acids was not tested, Holdeman *et al.* (1977) report that it is only necessary for Gram-negative organisms if they produce no major volatile acids which was not the case here. These 4 isolates identified as *Prevotella* spp. and with GLC profiles fitting could be expected to produce a major succinic acid peak if tested (Summanen *et al.*, 1993).

A FID (see section 1.8.4.6) was used in this study which cannot detect formic acid. *Actinomyces* spp., *Clostridium* spp. and *Eubacterium* spp. may produce the acid (Holdeman et al., 1977) but the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) does not include it.

It is possible to determine the amount of each substance within a sample, although this is unnecessary for identification purposes. Quantitative analysis is meaningless unless the researcher is certain of the identifications of all peaks (Drucker, 1981). A directly proportional relationship is seen between concentration of acid (volatile and non-volatile) either acidified or methylated and peak height (Holdeman *et al.*, 1977) therefore by measuring the heights of sample peaks and reading the values off a standard curve, an approximate concentration can be obtained (Holdeman *et al.*, 1977). This method relies on accurate and constant sample volumes (Drucker, 1981).

4.11.11 PARTIAL 16S RRNA GENE SEQUENCING

Partial 16S rRNA gene sequencing was used to suggest a closest phylogenetic relationship of unknown isolates by aligning partial sequences with those included within the EMBL and Genbank databases. The closest matches were checked against the Gram morphology, catalase test, oxidase activity and aerotolerance (other tests section 2.10.2 as relevant), if agreement was seen, this probable identification was accepted. Distance matrices were constructed (see appendicies section 6.10.3) for those isolates which were sequenced, in comparison with representative species of the genera to indicate the suitability of identifications.

As described in section 1.8.8.3, 16S rRNA is composed of conserved regions and variable regions which are species-specific (McDade and Anderson, 1996). The availability of rRNA gene sequences of previously characterised species allows identification of species by comparison (Busse *et al.*, 1996; McDade and Anderson, 1996; Tanner *et al.*, 1994). Partial

16S rDNA sequences were used during this study for identification purposes (Ludwig and Schleifer, 1994). The use of partial sequence analysis was quick and allowed systems already in place to be utilised and is valid for bacterial identification purposes (Ludwig and Schleifer, 1994).

Partial 16S rDNA sequence analysis of PLS isolates identified a wide range of species. An explanation of why certain isolates were studied in this way is given in section 3.12.3.2 (aerobes) and 3.13.3.2 (anaerobes). The majority were sequenced because the commercial kit identification was improbable in the light of other test results or there was no identification with a commercial kit and other tests either did or did not suggest a genus level identification. In these scenarios, sequence analysis was used to prove or provide a result. In a couple of cases, it was used to check an accepted identification.

Streptococcal species were identified (*S. anginosus*, *S. gordonii*, *S. intermedius*, *S. milleri* group and *S. sanguis*) with identities ranging from 85.6% (**6** *2B*; *S. milleri* group) to 99.6% (**11** *2A*; *S. anginosus*). The sequence obtained for **6** *2B*; *S. milleri* group had the lowest identity value of all the sequences. This is probably due to the determination of the sequence, which included a lot of degenerate bases where the sequence was not clear. Alternatively, it must not be ruled out that it may represent a more diverse species, which although a streptococci is as yet uncharacterised, especially due to the identification of *Lactococcus* obtained with the Rapid ID 32 Strep kit. Repeating the sequence analysis and if necessary looking at the entire 16S rRNA gene sequence would provide additional information needed to answer this query. The streptococci have been well characterised by 16S rRNA gene sequencing (Bentley *et al.*, 1991).

Comparison of 16S rRNA sequences from *L. cremoris*, *L. mesenteroides* and *L. paramesenteroides* reveals how similar they are. Indeed sequences from *L. cremoris* and *L. mesenteroides* differ only where the sequencing procedure raises sequence ambiguities, around 1460 bases. As a result, isolate **9** *2E*, has a high identity level to both species which makes it impossible to decide which species represents the correct identification. The distance data between **9** *2E* and *L. cremoris* and *L. mesenteroides* is considerably lower than between any other members of the genus used for comparison, suggesting that the genus identification is correct. *L. cremoris* represents a subspecies of *L. mesenteroides* (Handwerger *et al.*, 1990; Holzapfel and Schillinger, 1992), therefore an identification of *L. mesenteroides* applied to isolate **9** *2E* would be valid. It is impossible to say whether a full length 16S rRNA gene sequence would help identify the subspecies of this isolate.

Three isolates were identified as *Neisseria* species (*N. elongata* and *N. flavescens*). The sequences were compared to selected *Neisseria* species found in EMBL database and representing species of human (*N. elongata, N. flavescens, N. lactamica, N. polysaccharea*) and animal origin (*N. denitifricans*). Phylogenetic studies suggest that *N. elongata* and *N. denitifricans* are more closely related to each other than other *Neisseria* spp. (Tanner *et al.*, 1994). Entries for *N. cinerea* and *N. sicca* contain the terminal 800 bp and therefore could not be compared with the PLS sequences. The identification of **9** *2A* and **14** *2E* as *N. flavescens* was confirmed when distances data was examined which showed less dissimilarity between the PLS isolates and *N. flavescens* than seen between other members of the genus. This was also true of isolates **3** *2A* (*N. elongata*) and *N. elongata*. Identifications of *N. cinerea* were achieved for **9** *2A* and **14** *2E* using API NH and although a high level of similarity was seen between the sequences and *N. flavescens* in the database, the absence of *N. cinerea* sequences for comparison means this should not be considered absolute.

Two isolates were identified as *Kingella* species. One as *K. dentrificans* and *K. indologenes*, now called *S. indologenes* (see section 4.14), however the name is unchanged within the database. Analysis of nucleic acid distances was performed both including and excluding *K. indologenes* and with *K. denitrificans*, *K. kingae* and *K. oralis*. *S. indologenes* and isolate **3** *1E* had higher distance values i.e. more dissimilarity than seen amongst *Kingella* sp. Isolate **16** *1A* was confirmed as *K. denitrificans* with a distance value well below that between other species.

Two isolates were identified as *Actinomyces* sp. **3** *1A* as *Actinomyces* sp. and **1** *1B* as *A. israelii*. The levels of dissimilarity between the representative members of this genus were higher that seen amongst others tested, with distances between *A. israelii* and other species the greatest. Isolate **1** *1B* was very similar to *A. israelii*, displaying a lower distance value to one strain than seen between the two strains of *A. israelii* from the database, confirming the identification. Isolate **3** *1A* appeared more closely related to *A. israelii* and **1** *1B* (*A. israelii*) than other species, although the levels of difference are no smaller than seen between other species (*A. howelli*, *A. naeslundii*) and *A. israelii*. It has been noted that *A. israelii* may consist of two species (Schaal, 1992) which may explain this association of isolate **3** *1A* with *A. israelii*. Due to the differences seen amongst members of this genera, isolate **3** *1A* is most likely an *Actinomyces* species, although whether it represents a previously characterised one is unclear.

The partial sequence obtained for isolate **15** *1A* (*B. cereus*) is identical along its entire length with that of *B. cereus* contained within EMBL. It also displays limited differences to two other *B. cereus* sequences.

When sequences of *Peptostreptococcus* species were compared, low distance values were seen between *Pstr. anaerobius* than other strains including *Pstr. micros*. Isolate **11** *1A*, displays little similarity (in terms of distance value) to any other *Peptostreptococcus* strain compared. However, the values obtained between isolate **11** *1A* and other unspecified *Peptostreptococcus* are less than those seen between other sequences. This confirms that providing the taxonomic status of the isolates contained within the database is correct, isolate **11** *1A* is likely to be a *Peptostreptococcus*. GLC analysis of this isolate yielded iso-caproic acid suggesting *Pstr. anaerobius* (Murdoch and Mitchelmore, 1991).

As described in section 4.14.3.7, the genus *Stomatococcus* consists of a single species, *Stom. mucilaginosus*. For this reason, the partial sequence of isolate **4** *1C* II was not compared to any sequences, in addition, it displayed a 99.8% identity along its length to this species.

A discussion on partial 16S rRNA gene sequencing of *P. intermedia* and *P. nigrescens* is given in section 4.5.4.1.

Identifications of *Pseudomonas* sp.; *P. azotoformans* and *P. olveovorans* were achieved in 5 cases. *Pseudomonas* sp. consists of aerobic, Gram-negative rods which are catalase and oxidase positive and breakdown carbohydrates oxidatively, as determined by the oxidation-fermentation test (Cowan and Steel, 1993). In all cases, those isolates identified as *Pseudomonas* sp. were Gram-positive rods or cocci that were catalase and oxidase negative, 1 isolate was anaerobic whilst 4 out of the 5 were facultative anaerobes. It is accepted that some members of *Pseudomonas* are pathogenic and others may cause opportunistic infections (Collins *et al*, 1995). The author could find no evidence for a human habitat, or infection caused by *P. azotoformans* and *P. olveovorans* and the sources of the strains which have been sequenced was not given by the authors (Anzai *et al.*, 1997). However, a phylogenetic study of 16S rRNA gene sequences of *Pseudomonas* sp. clustered *P. olveovorans* with *P. mendocina* and *P. azotoformans* with *P. fluorescens*. *P. fluorescens* is an opportunistic pathogen of importance in cases of cystic fibrosis (Collins *et al.*, 1995) and *P mendocina* is found occasionally in clinical material (Cowan and Steel, 1993), therefore it is not unlikely that related species would be isolated from diseases of the immunocompromised. Despite this link, none of the isolates identified here as *Pseudomonas* fit the genus profile and this identification is discounted as contamination.

PCR-based sequencing methods are subjected to the same contamination concerns as all PCR techniques (Roux, 1995). UV light was used to decontaminate all PCR apparatus and reagents (Roux, 1995) but despite the use of UV irradiation, separate PCR and template DNA preparation areas and other sensible precautions (section 1.8.5.1), contamination still occurred. Post-PCR amplification using PCR products as a template provides the chance for further contamination to be introduced (Roux, 1995), by methods used to purify PCR template for re-amplification (Roux, 1995) and this is the most likely point at which the contamination was introduced. All reagents, used for the amplifications in question were discarded.

Unfortunately, it was not possible to repeat 16S rRNA gene sequencing on the contaminated isolates to determine whether the source of contamination came from the template, although, all cultures are checked for purity before DNA extraction.

The use of partial 16S rRNA gene sequencing for bacterial identification should not be necessary if enough suitable tests are performed. Where it was carried out, identifications were achieved. However, it must be remembered that the entire sequence length would be a more reliable indicator of sequence similarities or differences (see section 4.5.5 for a discussion about complete versus partial 16S rRNA gene sequencing). As only a partial sequence was used, some isolates cannot be identified beyond genera (**3** *1A - Actinomyces*) and cannot be thought of as new species or strains. It is unclear the extent of sequence differences required to consider isolates to be a new species (see section 4.5.6) and complete 16S rRNA gene sequences would be required for phylogenetic studies.

Not all isolates were sequenced and it is possible in the light of results for isolates **6** *2B* and **11** *2A* which had been previously identified as *Lactococcus* and *S. constellatus* respectively, that some identifications may be incorrect. If sequence analysis becomes routine for identification purposes, will the constantly changing taxonomy shift further or will we see the relationship between species and strain more clearly defined?

4.12 LOSS OF VIABILITY DURING IDENTIFICATION

Approximately 39% of all anaerobically cultivated isolates died before they could be identified compared to 20% of aerobic cultures. This is most likely due to a highly fastidious nature with growth requirements or oxygen tolerances which were not being met (Socransky *et al.*, 1987). Species such as *Actinomyces*, *Eikenella* and *Haemophilus* exhibit enhanced growth under conditions of increased carbon-dioxide and may be lost if incubated in an aerobic environment.

4.13 IDENTIFICATION OF BACTERIAL SPECIES - PUBLISHED PLS STUDIES

Microbiological methods for the identification of microorganisms from PLS patients are primarily cultural based (Clerehugh *et al.*, 1996; Eronat *et al.*, 1993; Ishikawa *et al.*, 1994), as was the case with this study although monoclonal antibody studies have been reported (Clerehugh *et al.*, 1996; Ishikawa *et al.*, 1994). Selective media is often employed using gentamicin to select for Gram-negative anaerobes (Clerehugh *et al.*, 1996) or for a specific range of pathogens such as *A. actinomycetemcomitans*, *Bacteroides*, *Veillonella* and streptococci (Eronat *et al.*, 1993), *Bifidobacterium*, *Eubacterium* and *Fusobacterium nucleatum* (Ishikawa *et al.*, 1994). The use of phase contrast microscopy (Ishikawa *et al.*, 1994) and scanning electron microscopy (Jung *et al.*, 1981; Rateitschak-Plüss, 1984) to detect spirochaetes and plaque composition has been reported. Cultivation of spirochaetes is not attempted.

Commercial identification kits have been used previously to provide an overview of all species within the oral cavity (Clerehugh *et al.*, 1996). That study successfully identified 33 species. The success rate of the identification kits used during this study was much lower (see section 4.10.1.2) and therefore the range of species identified smaller. Species such as *B. gracilis*, *Cardiobacterium hominis*, *H. aphrophilus*, *Selenomas* sp. and *Veillonella parvula* were identified (Clerehugh *et al.*, 1996) and represents one of the most comprehensive bacterial studies on PLS.

It seems that commercial identification kits provide the greatest range of identifications and perhaps used in parallel with a range of selective media (see section 4.9.2) would provide an adequate way of obtaining an overview of the subgingival species from these patients.

4.14 MICROBIOLOGY OF PLS

The use of identification studies to draw conclusions regarding the species associated with periodontal disease, in this case PLS, assumes that the species isolated are representative of all species present (Dahlén *et al.*, 1990a). No attempt has been made during this study to quantify any of the species isolated and therefore limited conclusions can be drawn about the prevalence or pathogenicity of any species isolated. Table 4.2 displays a complete list of the bacterial species isolated from two patients with PLS separated into aerobic, anaerobic, facultative and capnophilic species and representing 15 genera, including those species identified using monoclonal antibodies. Table 4.3 separates the species according to which patient they were isolated from allowing comparison between patients and tables 4.4 and 4.5 correlates species with the site that was sampled.

Gemella spp., *Neisseria* spp., *Peptostreptococcus* spp., *P. intermedia* and *Streptococcus* spp. were isolated from both patients. *Actinomyces* spp., *Bacillus* spp., *Capnocytophaga* spp., *Kingella* spp., including recently transferred *Suttonella indologenes* (Dewhirst *et al.*, 1990), *Mobiluncus* spp. and *Stomatococcus* spp. were isolated only from patient 1 and *P. gingivalis* was detected only in samples from patient 1. *Eubacterium* spp., L*euconostoc* spp. *P. nigrescens* and *P. melaninogenica* were isolated only from patient 2 and *A. actinomycetemcomitans* was detected only in samples from this patient. A larger number of isolates from patient 1 were identified compared to patient 2 which directly reflects a larger number of isolates received from MRI from patient 1. Whether this is the result of increased numbers of microorganisms in the oral cavity of patient 1, increased microflora of subgingival plaque from deciduous teeth in PLS patients, larger amounts of gingival material or more cultivable species is unknown.

4.14.1 OBLIGATELY AEROBIC SPECIES

4.14.1.1 *Bacillus* species

One isolate was identified as the Gram-positive spore former *Bacillus cereus*. As well as being an established cause of food-poisoning it is an opportunistic pathogen with recordings of eye and respiratory tract infections, meningitis, bacteraemia, septicaemia and wound infections following accidental trauma (for review see Logan, 1988).

Bacillus species are not considered part of the normal oral flora (Tanner *et al.*, 1994), although *B. cereus* has been reported in plaque samples (Tanner *et al.*, 1994). With no reports of an association to periodontal disease occurring with or without systemic disease, and due to its universal occurrence and the formation of spores, the possibility that this is a contaminant cannot be ruled out.

It is noted that the isolate identified as *B. cereus* (15 *1A*) was cultured under anaerobic conditions, whilst *B. cereus* is an aerobic species. As a consequence, *B. cereus* is listed as a facultative species.

Table 4.2 Complete list of bacterial species isolated from two patients with PLS

OBLIGATE AEROBE	*Neisseria* sp. *N. cinerea* *N. elongata* *N. flavescens* possibly *N. subflava*
OBLIGATE ANAEROBE	*Eubacterium* sp. *P. gingivalis* *Prevotella* sp. *P. intermedia* *P. melaninogenica* *P. nigrescens* possibly *P. oralis* *Peptostreptococcus* sp. *Pstr. anaerobius* *Pstr. micros*
FACULTATIVE	*A. actinomycetemcomitans* *Actinomyces* sp. *A. israelii* *B. cereus* *G. haemolysans* *G. morbillorum* *K. dentrificans* *Leuconostoc* sp. *L. mesenteroides* *Mobiluncus* sp. *Stom. mucilaginosus* *Streptococcus* sp. *S. anginosus* *S. bovis* II *S. constellatus* *S. gordonii* *S. intermedius* *S. milleri* group *S. mitis* *S. oralis* *S. salivarius* subsp. *salivarius* *S. sanguis* *S. indologenes*
CAPNOPHILIC	*Capnocytophaga* sp.

FOOTNOTE TO TABLE 4.2:

1. The list includes *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia* as identified using monoclonal antibodies (appendix 6.24).

Table 4.3 Complete list of bacterial species isolated from two patients with PLS, displaying association with patient

	PATIENT1	PATIENT 2
OBLIGATE AEROBES		
Neisseria sp.	✓✓	×
N. cinerea	✓	✓
N. elongata	×	✓
N. flavescens	×	✓✓
Neisseria sp. possibly *N. subflava*	✓	×
OBLIGATE ANAEROBES		
Eubacterium sp.	×	✓
Porphyromonas gingivalis	✓	×
Prevotella sp. possibly *P. oralis*	✓	×
P. intermedia	✓	×
P. melaninogenica	×	✓✓
P. nigrescens	×	✓✓
Peptostreptococcus sp.	✓	×
Pstr. anaerobius	×	✓
Pstr. micros	✓	×
FACULTATIVE		
A. actinomycetemcomitans	×	✓
Actinomyces sp.	✓	×
A. israelii	✓	×
B. cereus	✓	×
G. haemolysans	✓	×
G. morbillorum	✓	✓
K. dentrificans	✓	×
Leuconostoc sp.	×	✓
L. mesenteroides	×	✓
Mobiluncus sp.	✓	×
Stom. mucilaginosus	✓	×
Streptococcus sp. (to genus only)	✓	✓
S. anginosus	×	✓✓
S. bovis II	✓	×
S. constellatus	✓✓✓	×
S. gordonii	×	✓
S. intermedius	×	✓
S. milleri group	✓	✓
S. mitis	✓✓	✓
S. oralis	✓	×
S. salivarius subsp. *salivarius*	✓	✓
S. sanguis	✓✓✓	✓
Suttonella indologenes	✓	×
CAPNOPHILIC		
Capnocytophaga sp.	✓	×

KEY:

✓ Isolation of this species from PLS patient from one site, therefore number of ✓ = number of sites

× No isolation of this species from PLS patient from any site.

FOOTNOTE TO TABLE 4.3:

1. This records only presence or absence of an organism, no quantification studies were done.
2. The list includes *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia* as identified using monoclonal antibodies (appendix 6.24).

Table 4.4 Species isolated from PLS patient 1 separated according to site of sample

SITE A	
UPPER RIGHT DECIDUOUS CANINE	
Actinomyces sp.	*Peptostreptococcus* sp.
B. cereus	*Prevotella* sp.
Capnocytophaga sp.	*S. constellatus*
K. denitrificans	*S. mitis*
N. cinerea	*S. sanguis*
Neisseria sp.	*Streptococcus* sp.
SITE B	
LOWER LEFT DECIDUOUS CANINE	
A. israelii	*'S. milleri'* group
Mobiluncus sp.	*S. oralis*
Neisseria sp.	*S. sanguis*
S. constellatus	
SITE C	
UPPER LEFT DECIDUOUS 1ST MOLAR	
S. bovis II	*Stom. mucilaginosus*
S. constellatus	*N. subflava*
S. sanguis	
SITE D	
LOWER LEFT DECIDUOUS 1ST MOLAR	
G. haemolysans	*Pstr. micros*
G. morbillorum	*S. sanguis*
P. intermedia	
SITE E	
LOWER RIGHT DECIDUOUS CANINE	
S. indologenes	
SITE F	
LOWER RIGHT DECIDUOUS 1ST MOLAR	
S. mitis	*S. salivarius* subsp. *salivarius*

Table 4.5 Species isolated from PLS patient 2 separated according to site of sample

SITE A	
UPPER RIGHT PERMANENT 1ST MOLAR	
Eubacterium sp.	*P. nigrescens*
Leuconostoc sp.	*Pstr. anaerobius*
N. cinerea	*S. anginosus*
N. elongata	*S. intermedius*
N. flavescens	*S. salivarius* subsp. *Savlivarius*
P. melaninogenica	
SITE B	
UPPER RIGHT PERMANENT CANINE	
P. melaninogenica	*'S. milleri'* group
P. nigrescens	*S. mitis*
S. gordonii	*Streptococcus* sp.
SITE E	
LOWER RIGHT PERMANENT CANINE	
Leuconostoc mesenteroides	

4.14.1.2 *Neisseria* species

The aerobic species were predominantly *Neisseria* species, 7 isolates were identified as this species and all came from different sites in the 2 patients. *Neisseria* has been reported infrequently, *N. sicca/subflava* were isolated in 10 samples out of 19 by Clerehugh *et al.* (1996) and along with *S. sanguis* and *C. ochracea* represented the most commonly isolated species and *N. pharyngis* has been isolated by Preus (1988). In this study, identification of isolates as either *Neisseria* or *Prevotella* was second only to identification as *Streptococcus*. The taxonomy of the genus *Neisseria* is confusing due to conflicting biochemical information, and is complicated by taxonomic alterations amongst similar species in the genera *Branhamella*, *Moraxella* and *Kingella* (Barrett and Sneath, 1994). Aside from the pathogenic species *N. meningitidis* and *N. gonorrhoeae* (Morse and Knapp, 1992), the genus *Neisseria* contains strains (*N. mucosa*, *N. sicca* and *N. subflava* which includes those previously classified as *N. flava*, *N. perflava* and *N. pharyngis*; Barrett and Sneath, 1994) which are considered normal inhabitants of the nasopharynx (Johnson, 1983; Morse and Knapp, 1992) however they are opportunistic pathogens and have been isolated from patients with meningitis (especially *N. flavescens*), septicaemia and endocarditis (for review see Johnson, 1983).

N. cinerea, *N. elongata*, *N. flavescens* and *N. subflava* were isolated during this study. The species *N. sicca*, *N. subflava* and *N. mucosa* are early colonisers of a clean tooth surface (cited by Marsh and Martin, 1992) although isolation from periodontal disease is not commonly reported (Darveau *et al.*, 1997) the occurrence of *N. subflava* in the oral cavity of PLS patients is not surprising. The similarity of *N. flavescens* to other species especially *N. subflava* (Barrett and Sneath, 1994) or *N. cinerea* (Morse and Knapp, 1992) has been reported, suggesting that its occurrence in oral disease may have been underestimated due to misidentifications. The identifications of *N. flavescens* and *N. elongata* were made by partial 16S rRNA gene sequencing and its limitations for this must be considered (see section 4.5.5).

4.14.2 OBLIGATELY ANAEROBIC SPECIES

Four genera of obligately anaerobic bacteria were identified.

4.14.2.1 *Eubacterium* species

The genus *Eubacterium* is a genetically heterogeneous group (Tanner *et al.*, 1994) containing anaerobic non-spore-forming Gram-positive rods which are not similar to

Actinomyces, *Bifidobacterium*, *Lactobacillus* or *Propionibacterium* according to fermentation end-product analysis (Andreesen, 1992). *Eubacterium* species (including *E. brachy*, *E. nodatum* and *E. timidum*) have been isolated from subgingival plaque (Andreesen, 1992) and are associated with oral health, gingivitis (Darveau *et al.*, 1997) and periodontal disease in man (Moore *et al.*, 1987; 1991; Uematsu and Hoshino, 1992), but little evidence is available for an association with PLS. One study (Ishikawa *et al.*, 1994) failed to isolate any *Eubacterium* from gingival samples despite the use of selective media but low numbers were cultured from mouth rinse samples suggesting a presence in the oral cavity of PLS patients.

4.14.2.2 *Peptostreptococcus* species

Unidentified *Peptostreptococcus* spp. have been isolated from the subgingival pockets of patients with periodontal disease (Uematsu and Hoshino, 1992) and *Pstr. micros* has been associated with gingivitis (Darveau *et al.*, 1997). *Peptostreptococcus micros* (Clerehugh *et al.*, 1996) and unspeciated *Peptococcaceae* (Ishikawa *et al.*, 1994) have been identified from PLS patients. This study identified *Pstr. micros* in agreement with Clerehugh *et al.* (1996) and *Peptostreptococcus* sp. (Ishikawa *et al.*, 1994) from patient 1, in addition to *Pstr. anaerobius*, from patient 2.

4.14.2.3 *Porphyromonas* species

P. gingivalis is an accepted periodontal pathogen (Dahlén, 1993; Dzink *et al.*, 1988) which was detected here by monoclonal antibodies. The organism was detected in only one of the patients, which is either due to the sampling methods (Socransky *et al.*, 1987) or the true lack of this species. The use of monoclonal antibodies to *P. gingivalis* in a previous study (Clerehugh *et al.*, 1996) also failed to detect this species in 1 out of the 2 patients. It has been isolated from PLS patients previously by culture (Clerehugh *et al.*, 1996; Ishikawa *et al.*, 1994), but like *A. actinomycetemcomitans* (4.14.3.1) this recognised periodontal pathogen may not be important in PLS.

4.14.2.4 *Prevotella* species

Seven isolates were identified as *Prevotella*, of which 5 came from the same two sites from patient 2. *P. oralis*, *P. oris*, *P. loescheii* (Clerehugh *et al.*, 1996), *P. intermedia* (Clerehugh *et al.*, 1996; Ishikawa *et al.*, 1994) and other unspeciated black-pigmented anaerobes (D'Angelo *et al.*, 1992; Ishikawa *et al.*, 1994, Newman *et al.*, 1977) have been associated

with PLS. No mention is made of *P. nigrescens* among strains designated as *P. intermedia* although it is plausible that some would have been isolated, as two isolates from patient 2 were identified as *P. nigrescens* in this study. Both species have been associated with periodontal disease (Ashimoto *et al.*, 1996; Dzink *et al.*, 1985; Mättö *et al.*, 1996b; Savitt and Socransky, 1984), for further details see section 1.9.3. Whilst no work on PLS specifically mentions *P. melaninogenica*, it resides in the oral cavity and is a component of subgingival plaque (Shah, 1992) and has been associated with gingivitis (Dahlén, 1993) and periodontal disease (Savitt and Socransky, 1984). *P. intermedia* was detected by both culture and ELISA in this study, which are the same methods by which it has previously been associated with PLS (Clerehugh *et al.*, 1996).

4.14.2.5 Spirochaete species

Unspeciated spirochaetes were seen within gingival smears during this study. Spirochaetes are commonly seen in dental plaque from both gingivitis, adult periodontitis (Savitt and Socransky, 1984; Listgarten, 1976; Loesche and Laughon, 1982) and juvenile periodontitis (Savitt and Socransky, 1984), they are frequently unspeciated but detected by microscopy (Loesche *et al.*, 1985; Savitt and Socransky, 1984). This is probably due to their reputation as difficult to culture (Loesche and Laughon, 1982). The presence of unspeciated oral spirochaetes has been reported previously in PLS patients (Ishikawa *et al.*, 1994; Jung *et al.*, 1981; Rateitschak-Plüss and Schroeder, 1984).

4.14.3 FACULTATIVE ANAEROBES AND MICROAEROPHILIC SPECIES

Facultative species represent the largest group and the majority of those identified were streptococci.

4.14.3.1 *Actinobacillus actinomycetemcomitans*

A. actinomycetemcomitans is an accepted periodontopathogen (Darveau *et al.*, 1997; Dzink *et al.*, 1988) which has been associated particularly with prepubertal periodontitis (Delaney and Kornman, 1987) and localised juvenile periodontitis (Zambon *et al.*, 1983). It has been associated with PLS on many occasions (see section 1.7.; Bimstein *et al.*, 1990; Clerehugh *et al.*, 1996; Eronat *et al.*, 1993; Ishikawa *et al.*, 1994; Van Dyke *et al.*, 1984) and is considered an important pathogen for the periodontal component of PLS (Preus and Gjermo, 1987). During this study it was detected by monoclonal antibodies but only in one sample from patient 2, this does not correlate with current opinion regarding its importance.

Detection via monoclonal antibodies discounts the need for cultivation, thus the viability of cells is not important. For this reason, it is fair to assume *A. actinomycetemcomitans* did not occur in high numbers in either of the patients studied here and therefore the presence of accepted periodontopathogens may not be necessary for PLS to progress. This argument could also apply to *P. gingivalis* which was only detected in patient 1. In contrast to the ELISA result, raised antibody levels to *A. actinomycetemcomitans* have been detected in two patients studied previously (Ishikawa *et al.*, 1994).

4.14.3.2 *Actinomyces* species

Three isolates were identified as *Actinomyces* species and all three isolates came from patient 1 and two of the isolates were isolated from the same site. Although *Actinomyces* have been associated with PLS (Clerehugh *et al.*, 1996) the species (*A. israelii*) identified here has not been specifically mentioned. *Actinomyces* sp. form part of the resident oral microflora of man (Schaal, 1992) and are therefore isolated from the healthy oral cavity. They are associated with gingivitis (Darveau *et al.*, 1997; Page, 1986), especially *A. israelii* and *A. naeslundii*.

4.14.3.3 *Gemella* species

The genus *Gemella* consists of two species previously belonging to other genera; *G.* (*Neisseria*) *haemolysans* and *Gemella* (*Streptococcus*) *morbillorum* (Bascomb and Manafi, 1998). Both species have been associated with endocarditis (Martin *et al.*, 1995; Samuel *et al.*, 1995) but form part of the normal human flora (Facklam and Elliott, 1995); *G. haemolysans* in the oropharynx and *G. morbillorum* in the gastrointestinal tract. *G. morbillorum* has been isolated from PLS patients and is also associated with a healthy mouth (Darveau *et al.*, 1997), whilst no association with periodontal disease or PLS has been reported for *G. haemolysans*. The likelihood of *Gemella* causing similar infections to those caused by viridans streptococci has been noted (Facklam and Elliott, 1995), therefore identification from these patients is not too surprising.

4.14.3.4 *Kingella* species

Kingella species consists of *K. denitrificans*, *K. kingae* (Swann and Holmes, 1984) and *K. oralis* (Dewhirst *et al.*, 1993, 1994). One species *K. indologenes*, has been transferred to a new genus as *Suttonella indologenes* (Dewhirst *et al.*, 1990). Despite being known upper respiratory tract commensals (Swann and Holmes, 1984), there are no reports of isolation

of members of this genus from PLS patients and only limited associations with periodontal disease. The primary habitat of *K. oralis* is human dental plaque (Dewhirst *et al.*, 1993, 1994) and it been isolated from supra- and subgingival plaque taken from both healthy oral cavities and patients with periodontitis (Chen, 1996). *K. denitrificans* and *K. indologenes* were identified during this study, both by the closest relationships to the partial 16S rRNA gene sequence. *K. kingae* has been reported to cause endocarditis in patients with underlying heart disease (for review see Morrison and Wagner, 1989) as has *K. denitrificans* (Swann and Holmes, 1984), although the latter has also been found in the supragingival plaque of a patient with juvenile periodontitis (Dewhirst *et al.*, 1993). The isolate identified as *K. indologenes* should be renamed *S. indologenes* (Dewhirst *et al.*, 1990).

4.14.3.5 *Leuconostoc* species

The genus *Leuconostoc* contains the species *L. mesenteroides* (including subsp. *cremoris* and subsp. *dextranicum*), *L. paramesenteroides*, *L. lactis* and *L. oenos* (Handwerger *et al.*, 1990). They are similar to streptococci and their initial identification as *S. sanguis* type II as well as *S. salivarius* and *S. pneumoniae*, using automated identification systems has been reported (Handwerger *et al.*, 1990). Production of carbon-dioxide from glucose and resistance to vancomycin will distinguish *Leuconostoc* from the viridans streptococci (includes those species frequently isolated from the mouth) and the Gram morphology will distinguish them from lactobacilli (Handwerger *et al.*, 1990). In the event that *Leuconostoc* is seen as coccobacilli, it can be distinguished from heterofermentative lactobacilli by the inability to hydrolyze arginine (Holzapfel and Schillinger, 1992). There is no historical evidence for association of this species with PLS, however the occurrence of members of this species in the blood has been reported in patients already critically ill with diseases including acute leukaemia, renal failure and HIV infection (Handwerger *et al.*, 1990) supporting the isolation of the species from these patients. It has been suggested that the incorrect identification of *Leuconostoc* as viridans streptococci means that the pathogenic associations of this species have been underestimated (Handwerger *et al.*, 1990) and suggests that further investigation of any viridans streptococci or *S. sanguis* isolated may be required. During this study both viridans streptococci (*S. constellatus*, *S. mitis*, *S. oralis* and *S. sanguis*) and *S. salivarius* were identified using Rapid ID 32 Strep, further work would be required to check that none of these isolates was *Leuconostoc* sp. (Handwerger *et al.*, 1990).

4.14.3.6 *Mobiluncus* species

The genus *Mobiluncus* was first described by Spiegel and Roberts (1984) as anaerobic motile curved rods, contains the species *M. curtisii* (shorter rods) and *M. mulieris*, isolated from patients with bacterial vaginosis (Spiegel and Roberts, 1984). *Mobiluncus* has been isolated from extravaginal sources (Spiegel, 1992), however, the author could find no evidence of isolation from oral sources. It should be considered that this species may be misidentified as *Actinomyces* due to a positive Gram strain and a similar GLC profile (Spiegel, 1992) which would underestimate their occurrence.

4.14.3.7 *Streptococcus* species

Ten streptococcal species were identified including two isolates identified only as belonging to the *S. milleri* group, which includes the species *S. anginosus*, *S. constellatus* and *S. intermedius* (Whiley and Beighton, 1991). All three species of the '*S. milleri* group' were isolated during this study. Of the streptococcal species identified here, *S. mitis*, '*S. milleri* group' and *S. sanguis* (Clerehugh *et al.*, 1996) have been previously associated with PLS and studies by both Eronat *et al.* (1993) and Ishikawa *et al.* (1994) mention the occurrence of streptococci without reference to species. Of the other streptococcal species identified during this study, there is no recorded involvement of *S. gordonii* and *S. oralis* with PLS although both have been isolated from the healthy oral cavity (Moore *et al.*, 1991) and *S. oralis* has been associated with gingivitis (Darveau *et al.*, 1997). As well as forming part of the healthy oral microflora and occurring in gingivitis (Darveau *et al.*, 1997), the '*S. milleri* group' has been associated with brain abscesses, liver abscesses and ear, nose and throat infections (for review see Gossling, 1988) whilst members of the *S. oralis* group of viridans streptococci (*S. gordonii*, *S. oralis*, *S. sanguis*) have been associated with infective endocarditis (Douglas *et al.*, 1993). *S. salivarius* was also identified during this study and has been associated with oral health (Moore *et al.*, 1991). *S. bovis* inhabits the gastrointestinal tract and is associated with colon cancer and bowel disease (Ruoff, 1992). It consists of two biotypes; type I produces dextran whilst type II does not produce EPS (Ruoff, 1992). Biotype II is frequently associated with infective endocarditis and has been isolated from cerebrospinal fluid of patients with meningitis (Cohen *et al.*, 1997). Although *S. bovis* was isolated from PLS patients the author could find no evidence which readily suggests an association with periodontal disease. It has been noted that researchers should expect to isolate microorganisms associated with oral health in studies of periodontal

disease, despite that fact that they are mostly overgrown by disease associated species (Moore *et al.*, 1991).

The taxonomy of streptococcal species is complicated, although an understanding is essential for identification and it is summarised below. Historically members of the genus were grouped as enteric, lactic, pyogenic or viridans (Bascomb and Manafi, 1998) before the creation of *Enterococcus* and *Lactococcus*. This was followed by splitting remaining species into 6 groups (Bentley *et al.*, 1991; Bascomb and Manafi, 1998);

(1) pyogenic, including *S. pyogenes*, *S. agalactiae*, *S. zooepidemicus* and animal strains; (2) group D antigen streptococci, *S. bovis* and *S. equinus*; (3) viridans streptococci consisting of the *S. milleri* group - *S. anginosus*, *S. constellatus* and *S. intermedius* and the *S. oralis* group - *S. sanguis*, *S. gordonii*, *S. parasanguis*, *S. mitis*, *S. oralis* (Bascomb and Manafi, 1998); (4) *S. mutans* group including *S. sobrinus*, *S. cricetus*, *S. rattus* and *S. macacae*; (5) *S. salivarius* group - *S. salivarius* and *S. vestibularis* (cited by Marsh and Martin, 1992) and (6) other unaffiliated species *S. acidominimus* and *S. suis* (Bascomb and Manafi, 1998). The *S. mutans*, *S. milleri*, *S. oralis* and *S. salivarius* groups represent oral streptococci.

4.14.3.8 *Stomatococcus* species

One isolate was identified as *Stomatococcus mucilaginosus*, an organism found within the oral microflora (Ascher *et al.*, 1991; Bergan and Kocur, 1982). *S. mucilaginosus* has not been previously associated with the periodontal component of PLS. Its occurrence has been reported in blood infections of immunocompromised patients including bone marrow transplant patients and those with acute leukaemia (McWhinney *et al.*, 1992) often related to the presence of central venous catheters (Ascher *et al.*, 1991) and endocarditis following insertion of prosthetic valves (Ascher *et al.*, 1991). The variable catalase reaction can delay identification (McWhinney *et al.*, 1992) and cause confusion between staphylococcal and streptococcal species. Differentiation of this organism from streptococcal species can be achieved by observation of grey mucoid colonies (McWhinney *et al.*, 1992).

4.14.4 CAPNOPHILIC SPECIES

4.14.4.1 *Capnocytophaga* species

The genus *Capnocytophaga* consists of 5 species known to inhabit humans; *C. gingivalis*, *C. granulosa*, *C. haemolytica*, *C. ochracea* and *C. sputigena* (Yamamoto *et al.*, 1994). Members of the genus *Capnocytophaga* are frequently associated with gingivitis (Darveau

et al., 1997), periodontal disease (Dzink *et al.*, 1988) and juvenile periodontitis (Savitt and Socransky, 1984). Studies have also reported *Capnocytophaga* in increased numbers where there is a lower subgingival temperature and therefore fewer accepted periodontopathogens, reflecting a decreased risk of periodontal disease (Haffajee *et al.*, 1991). These species are reported commonly from PLS patients (Clerehugh *et al.*, 1996; Eronat *et al.*, 1993; Ishikawa *et al.*, 1994; Tinanoff *et al.*, 1986), *C. ochracea* (Clerehugh *et al.*, 1996) has been isolated but the majority of studies cite unspeciated *Capnocytophaga*. No attempt was made to speciate the *Capnocytophaga* isolates identified, although techniques (MLEE; Frandsen *et al.*, 1996 and DNA probes; Conrads and Brauner, 1995) are reported that will.

4.14.5 SPECIES THAT WERE NOT ISOLATED DURING THIS STUDY

Fusobacterium spp. (Clerehugh *et al.*, 1996; Tinanoff *et al.*, 1986), *P. gingivalis*, *A. actinomycetemcomitans* and *Veillonella* (Clerehugh *et al.*, 1996; Ishikawa *et al.* (1994) often associated with PLS were not found by culture in this study. *Eikenella corrodens* has been isolated from PLS patients by numerous researchers using cultivation (Clerehugh *et al.*, 1996; Ishikawa *et al.*, 1994; Tinanoff *et al.*, 1986) and ELISA (Ishikawa *et al.*, 1994). Other species not so often reported including *P. loescheii* and *B. gracilis* (Clerehugh *et al.*, 1996) were not seen during this study.

It is possible that these anaerobic species were not identified because they were not present, however, it is also likely that they were not seen due to practical reasons, such as failure to maintain an anaerobic environment during transport (Dahlén *et al.*, 1993) or highly fastidious growth requirements (Socransky *et al.*, 1987). One isolate (**10** *2A*, anaerobic) gave a 3% identity level with *Fusobacterium* when tested with the Rapid ID 32 A kit, the Gram morphology was typical of *Fusobacterium* but it died before further work could be done. Loss of viability *in vivo* could account for many unseen species. *P. gingivalis* and *A. actinomycetemcomitans* were not isolated by culture but were detected in subgingival plaque samples by monoclonal antibodies. *P. intermedia* was also detected by this method.

4.15 AN ALTERNATIVE APPROACH TO IDENTIFICATION

As already stated (section 4.9), all the primary examination of specimens was done by the diagnostic laboratory at MRI and the way in which identifications were obtained has been described. Below is a description of the way in which clinical samples for identification would have been handled in the absence of help from the diagnostic laboratory at MRI, and if the initial aim of the study had been different and the time constraints more relaxed.

The *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) suggests that the first stage in isolation and identification procedures is a direct examination by Gram stain or phase-contrast microscopy, of a clinical sample, to provide an indication of the kinds of species present due to characteristic cell morphology. The sample should be inoculated onto appropriate medium both selective and nonselective, and incubated aerobically and anaerobically. For an anaerobic environment and selection of obligate anaerobes, the use of a nonselective blood medium (FAA, CBA or Brucella agar) is recommended (Summanen *et al.*, 1993) in addition to the selective media Bacteroides bile esculin agar for *B. fragilis* and *Bilophila* (Summanen *et al.*, 1993), kanamycin-vancomycin laked blood agar and phenylethyl alcohol sheep blood agar (Summanen *et al.*, 1993) the specificities of these media are given in the introduction section 1.8.1. Blood agar (CBA or Brucella agar), chocolate agar, MacConkey agar and phenylethyl alcohol sheep blood agar are suggested for the recovery of aerobic bacteria (Summanen *et al.*, 1993). Incubation of a blood or chocolate agar plate in an atmosphere of 5-10% carbon dioxide may be considered so that capnophilic or microaerophilic species such as *Haemophilus* (section 4.9.4) are not lost (Summanen *et al.*, 1993). Note that *Actinomyces*, *Bifidobacterium* and *Lactobacillus* will also grow in a CO_2 enriched atmosphere (Summanen *et al.*, 1993).

Section 1.8 of the introduction details the approaches of two texts to sample identification. Probably, a combination of both approaches would be suitable. Primary isolation plates would be prepared as described above and on examination of colonies each different colony would be carefully described (as covered in section 4.9.3) and subcultured to give pure cultures. All pure colonies would be Gram stained. This would be followed by tests suggested by both Summanen *et al.* (1993) and Cowan and Steel (1993); catalase and oxidase production, aerotolerance, spore formation (Gram-positive isolates only), acid production from glucose metabolism, the breakdown of carbohydrates and the use of special potency disks (see below) for anaerobes. Enough information would be generated from these tests to ensure the correct identification kits were selected for use and commercial kits (API, RapID ANA-II) and Gas-Liquid Chromatography (GLC) would be performed next. Tests which can be used for more detailed species identifications could be adopted from Cowan and Steel (1993) or the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) as necessary such as lipase and lecithinase production for suspected *Clostridium* spp. (Summanen *et al.*, 1993) or nitrate and nitrite reduction and resistance to 20% bile for *B. fragilis* (Summanen *et al.*, 1993). Routine sequence analysis

should not be necessary for identification but could be used to add weight to any questionable identifications or for organisms of special interest (see section 4.11.11).

With the exception of optochin resistance (an antibacterial), no work was done to determine antibiotic sensitivities which can be of use for identification studies, for example the vancomycin resistance of *Leuconostoc* spp. (Handwerger *et al.*, 1990). The *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al*, 1993) describes the use of special potency disks of kanamycin (1000 μg), vancomycin (5 μg) and colistin (10 μg) to group anaerobes in the early stages of identification. Kanamycin selects for the resistant *Prevotella* and *Porphyromonas* species as well as *B. fragilis* group organisms, vancomycin selects for Gram-negative rods including *Fusobacterium* spp. but omitting *Porphyromonas* spp. and colistin allows growth of Gram-positive species, *Porphyromonas* spp. and *B. fragilis* (Summanen *et al.*, 1993). In addition to these, the use of a sodium polyanethol sulfonate disk will separate *P. anaerobius* from other, resistant Gram-positive cocci (Summanen *et al.*, 1993). Many studies detail identification schemes for groups of bacteria including viridans streptococci (Kikuchi *et al.*, 1995) *Lactococcus*, *Leuconostoc*, *Gemella* (Facklam and Elliott, 1995) and peptostreptococci (Murdoch and Mitchelmore, 1991).

4.15.1 NUCLEIC ACID PROBES

An overview of the range of methods available for the identification of periodontal pathogens from oral samples is discussed in section 1.8. Instead of traditional methods, the species isolated here could have been detected by nucleic acid probes (see section 1.8.7 and 1.10.12 for *P. intermedia* and *P. nigrescens*) obviating the need for cultivation and the possibility of losing fastidious organisms (Albandar and Olsen, 1990) and hastening the time from sampling to identification (Tenover, 1988). Whole genomic probes have been reported against *A. actinomycetemcomitans*, *B. forsythus*, *C. gingivalis*, *C. ochracea*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *Pstr. micros*, *S. intermedius*, *S. gordonii and S. oralis* (Paster *et al.*, 1995), *B. forsythus* (Lotufo *et al.*, 1994), *Campylobacter recta*, *E. corrodens* and *F. nucleatum*, (Lippke *et al.*, 1989). Oligonucleotide probes against *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *P. nigrescens* (Conrads and Brauner, 1995) *C. gingivalis*, *C. ochracea*, (Conrads *et al.*, 1996) and *Mobiluncus* sp. (Tiveljung *et al.*, 1996) and cloned probes against oral spirochaetes (DiRienzo *et al.*, 1991) have been described. Theoretically all microorganisms possess sequences unique to a species, conserved throughout the species, that can be exploited to produce a probe for subsequent detection of that species (Eisenstein, 1990).

4.16 A SUMMARY OF BACTERIAL IDENTIFICATION

There are many excellent texts providing full details of methods used to identify bacterial isolates. The *Anaerobe Laboratory Manual* (Holdeman *et al.*, 1977) and *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) deal in depth with anaerobic bacteria including sampling and culture procedures and are invaluable to the anaerobic microbiologist. Collins and Lyne's *Microbiological Methods* (Collins *et al.*, 1995) and Cowan and Steel's *Manual for the Identification of Medical Bacteria* (Cowan and Steel, 1993) cover all common staining and testing procedures, while Cowan and Steel (1993) lead the user step by step through a serious of tables which gradually refine the identification of clinical isolates, Collins *et al.* (1995) give a general guide to practical microbiology and cover food, environmental and water microbiology as well as medical microbiology and providing the necessary information for identification of isolates.

These guides tend to offer more than one method to perform particular tests, and although they have not all been covered in this thesis, there are many tests (used alone or as supplements to commercial identification kits, which can be used to give satisfactory identifications without the need for molecular or immunological techniques.

Identification of microorganisms within the oral cavity is a time consuming task (Slots, 1986b). It is complicated by the large numbers of bacterial species within the oral cavity (Moore *et al.*, 1985) many of which require different environments and media for successful isolation (Socransky *et al.*, 1987). Other methods for identification including oligonucleotide probes, enzyme and protein electrophoretic profiles and serological techniques are in use but they are not always readily available or applicable to all taxa and still rely on the fact an organism has been characterised by cultural studies (Socransky *et al.*, 1987). The use of culture and biochemical testing remains an important practice, with the constant potential to isolate uncharacterised species overlooked by other methods and allowing antibiotic sensitivity testing (Slots, 1986b). This study has attempted to look at the potential of combining traditional cultivation and identification techniques with rapid characterisation kits and the newer approaches of monoclonal antibodies and partial 16S rRNA gene sequencing. As a result, identifications vary in the amount of information gained and the reliability, based on the limitations of computer databases (API kits; section 4.10.1.1 and available 16S rRNA gene sequences).

4.17 IDENTIFICATION OF CLINICAL ISOLATES IDENTIFIED AS *PREVOTELLA INTERMEDIA* BY RAPID ID 32 A

A range of techniques were used to examine the 4 black-pigmenting clinical isolates A-D. All techniques have been discussed in detail previously. Neither the commercial identification kits RapID ANA II system and Rapid ID 32 A, traditional laboratory tests nor GLC can differentiate between *P. intermedia* and *P. nigrescens*.

4.17.1 TECHNIQUES WHICH DO NOT DIFFERENTIATE *P. INTERMEDIA* FROM *P. NIGRESCENS*

4.17.1.1 Commercial identification kits

The test results are variable between the two species (3.15.1), especially when using Rapid ID 32 A. Results of mannose and raffinose fermentation (MNE; RAF), phosphatase alcaline (PAL), alpha fucosidase (αFUC) and hydrolysis of arginine, histidine and glutamyl acid (ArgA; HisA; GGA) gave no consistent result for all 4 isolates. In particular, MNE, RAF and ArgA were positive for both isolates A and D (*P. nigrescens*) which was not true for the *P. intermedia* isolates. Further strains of each species would need to be examined to determine whether these tests are always positive for *P. nigrescens*, however it is interesting to note positive results of MNE and RAF tests for PINLO HST 1156, HST 2160, *P. corporis* strains and *P. pallens* strains and a positive ArgA result for all other species and strains tested (see table 6.23, appendix 6.23). Variable αFUC activity had been reported previously among strains of *P. nigrescens* (Shah and Gharbia, 1992a) and is seen here (isolate A negative, isolate D positive). In addition, a strong positive β-glucosidase (βGLU) has been reported for *P. nigrescens* (Shah and Gharbia, 1992a), which was not reported here using either Rapid ID 32 A or RapID ANA II system, although a positive α- glucosidase (αGLU) result was seen (Summanen *et al.*, 1993).

As stated above, variable αFUC activity has been reported (Shah and Gharbia, 1992a) and it is interesting to note that isolate A which was αFUC negative using Rapid ID 32 A was αFUC positive when tested with RapID ANA II. It is unclear whether this is a true reflection of variable strain activity or the result of incorrect test performance or interpretation. This could be resolved by a large study encompassing many strains of *P. intermedia* and *P. nigrescens*. This inconsistency between the results obtained by both types of anaerobe identification kit (3.15.1) is also seen for arginine hydrolysis (Arg A, Rapid ID 32 A; ARG, Rapid ANA II).

P. intermedia and *P. nigrescens* and *Bacteroides intermedius* historically, have been the subjects of limited studies evaluating identification kits (Celig and Schreckenberger, 1991; Dellinger and

Moore, 1986; Marler *et al.*, 1991). In each case, the kit of choice has been RapID ANA II and correct identification of either *P. intermedia* (*B. intermedius*) was made in the majority of (Celig and Schreckenberger, 1991), if not all, cases (Dellinger and Moore, 1986; Marler *et al.*, 1991). The inability of identification kits to distinguish the two species was reported in 1986 (Dellinger and Moore) as the inability to differentiate the two homology groups of *B. intermedius*.

4.17.1.2 Traditional identification tests

P. intermedia and *P. nigrescens* are catalase negative (Fukushima *et al.*, 1992; Summanen *et al.*, 1993), no difference in the catalase activity between the species has been reported previously and this was confirmed here. As described in section 1.19.2.1 of the introduction, conflicting information concerning the lipase reaction exists. A slower lipase positive reaction has been reported for *P. nigrescens* than *P. intermedia* (Shah and Gharbia, 1992a). Although this was not seen here, the lipase reactions appeared after 24 h for both species, therefore both *P. intermedia* and *P. nigrescens* were positive (Pearce *et al.*, 1996; Shah and Gharbia, 1992a).

4.17.1.3 GLC

Analysis of volatile fermentation end-products by both *P. intermedia* and *P. nigrescens* yielded acetic acid, iso-butyric and iso-valeric acids as previously reported (Shah and Gharbia, 1992a). Both species also produce succinic acids (Shah and Gharbia, 1992a), although the formation of methyl esters of non-volatile acids was not tested (sections 2.11.3.3.5 and 4.11.10; Holdeman *et al.*, 1977). Quantitative studies were not undertaken, therefore the work by Takada and Hirasawa (1997) cannot be substantiated.

4.17.2 TECHNIQUES WHICH DIFFERENTIATE *P. INTERMEDIA* FROM *P. NIGRESCENS*

Detailed discussions of these techniques can be found in sections 4.4 (RAPD-PCR), 4.5 (partial 16S rRNA gene sequencing) and 4.6 (PCR), each technique is suitable for species differentiation.

4.17.2.1 RAPD-PCR

All four isolates were provisionally identified as *P. intermedia* until subjected to RAPD-PCR (section 2.5.8). The isolates behaved in the same way as known strains of the two species with amplified bands I and III or II and IV. Although very similar, the RAPD-PCR amplification profile differs slightly between A and D and it is interesting to note that both

of these isolates came from patient 2. This suggests that there may be a high level of intra-individual homogeneity among oral strains, as suggested previously (Mättö *et al.*, 1996) by ribotyping which determined typically only 1-2 ribotypes or either (both) species from any individual, although up to 4 ribotypes were seen. Slightly more differences are seen between B and C and these *P. intermedia* isolates come for different patients.

4.17.2.2 Partial sequencing of 16S rRNA gene

Partial sequencing of 16S rRNA gene successfully identified isolates A-D and confirmed species identification as determined by RAPD-PCR. Sequence distance data suggests a higher degree of similarity between isolates A and D (*P. nigrescens*) than between B and C (*P. intermedia*). This is in agreement with similarity values determined by cluster analysis of RAPD-PCR profiles.

4.17.2.3 PCR with *P. intermedia* and *P. nigrescens* specific primers

Although isolates A-D were successfully identified by the appropriate species-specific primers (see section 4.6.7), amplification by these primers of other strains was inconsistent and in some cases non-specific. This rules out this technique as suitable for species differentiation until further studies have been conducted.

4.18 FURTHER EXAMINATION OF PINLOS

During this study, three PINLO isolates which did not fit the new description of PINLO (Könönen *et al.*, 1998a) were examined in comparison with *P. corporis*, *P. intermedia*, *P. nigrescens* and *P. pallens* using techniques discussed fully in sections 4.4, 4.5, 4.6, 4.10 and 4.11. These strains originate from non-oral sources (Devine *et al.*, 1994) but are identified as *P. intermedia*.

4.18.1 TECHNIQUES WHICH DO NOT DISTINGUISH PINLOS FROM *P. INTERMEDIA* OR *P. NIGRESCENS*

4.18.1.1 Commercial identification kits RapID ANA II system and Rapid ID 32 A

The PINLOs were identified as *P. intermedia* using both Rapid ID 32 A and the RapID ANA II System. Comparison of the profiles with strains of *P. corporis*, *P. intermedia*, *P. nigrescens* and *P. pallens* revealed slight differences. *P. pallens* were included due to reclassification of some PINLOs as *P. pallens* (Könönen *et al.*, 1998a). The test results for *P. pallens* were not much different from those previously described (Könönen *et al.*,

1998a); positive α- glucosidase (αGLU) with both kits, negative α-galactosides (both kits) and β-galactosidase (Rapid ID 32 A), negative β-glucuronidase (Rapid ID 32 A) and β-N-acetyl-glucosaminidase. Positive αFUC tests have been reported (Könönen *et al.*, 1998a), although this was seen for *P. pallens* strain AHN 9423 only. The profiles produced by PINLOs displayed no αFUC activity with either kit, agreeing with earlier findings for HST 1156 or HST 2160 (Devine *et al.*, 1994). PINLOs were also negative for phosphatase alcaline (PAL) with both kits. This is another distinction between PINLOs and *P. pallens* which are positive (Könönen *et al.*, 1998a), although negative PAL tests were seen when *P. pallens* were tested using RapID ANA II. *P. corporis* is also identified as *P. intermedia* by Rapid ID 32 A and the lack of this species within the Rapid ID 32 A database is recorded (Moll *et al.*, 1996). *P. corporis* is found within the RapID ANA II database, and is correctly identified (Dellinger and Moore, 1986) as confirmed by this system. Like *P. intermedia*, *P. nigrescens* and *P. pallens*, PINLOs were indole positive using both kits, *P. corporis* was indole negative as expected (Summanen *et al.*, 1993).

The combined results from the Rapid ID 32 A kit and Rapid ANA II suggests that the PINLOs tested here are not *P. pallens* and the indole positive reaction may be confirmation of a closer relationship to *P. intermedia* and *P. nigrescens* than *P. corporis*.

4.18.1.2 Traditional laboratory identification tests

Although the Gram morphology, anaerobic metabolism and catalase activity was consistent with *Prevotella*, the appearance of PINLOs on blood-FAA confirmed that these species were not *P. pallens*. *P. pallens* are weakly pigmenting and appear as tan to light brown colonies on blood agar (Könönen *et al.*, 1998a), the PINLOs grew as black colonies.

4.18.1.3 GLC

P. pallens produces acetic, iso-valeric and succinic acids (Könönen *et al.*, 1998a) from glucose metabolism and *P. corporis* produces acetic, iso-butyric, iso-valeric, succinic acids (Summanen *et al.*, 1993), the same as *P. intermedia* and *P. nigrescens*. Although the two *P. pallens* strains were not studied by GLC during this work, the PINLO strains were, and all three produced large iso-butyric peaks in addition to acetic and iso-valeric acids. This is further evidence of the lack of association to *P. pallens* and suggests an identification of the PINLOs as *P. intermedia*, as previously reported for HST 1156 or HST 2160 (Devine *et al.*, 1994).

4.18.2 TECHNIQUES WHICH DO DISTINGUISH PINLOS FROM *P. INTERMEDIA* AND *P. NIGRESCENS*

4.18.2.1 RAPD-PCR

As discussed in section 4.4.9.2, the RAPD-PCR amplification profiles of PINLOs are different from *P. intermedia* and *P. nigrescens* and also from *P. corporis* and *P. pallens*. Comparison of banding patterns was limited during this study, although there was the potential for a larger number of *P. intermedia* and *P. nigrescens* strains to be compared it was not possible to compare figure 3.8 with either figure 3.10 or 3.11. For accurate identification all strains for identification must be compared on the same gel due to errors introduced when the distances of the fragments are measured and the molecular weights determined (Baleiras Couto, 1995). Despite this, enough information is available to draw some limited conclusions. Initial comparison of the PINLOs with *P. intermedia* and *P. nigrescens* shows that the PINLOs do not amplify the species-specific marker bands I-IV. However, closer analysis shows that all three strains did amplify the *P. intermedia* specific band number II and that strain A391 also displays a low intensity band IV (also *P. intermedia*). Further work is required to determine whether bands II and IV cannot be considered *P. intermedia*-specific markers or whether strain A391 is a strain of *P. intermedia* or a subspecies. The latter two options seem unlikely, as MLEE failed to allocate either HST 1156 or HST 2160 to *P. intermedia* or *P. nigrescens* (Devine *et al.*, 1994) and in addition, cluster analysis of banding patterns reveals only a 62% similarity between the profiles of A391 and that of *P. intermedia* ATCC 25611. A 62% similarity is also seen between *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 25261 and ATCC 33563 which possibly infers a species level relationship between *P. intermedia*, *P. nigrescens* and PINLOs. Analysis of the banding patterns of a larger number of strains are required to add any statistical significance to these findings and determine the specificity of bands II and IV.

It must be remembered that RAPD-PCR is an analysis of the entire genome and bands II and IV may represent areas of homologous sequence between PINLO and *P. intermedia*. All three PINLO strains amplify bands which were not seen from *P. intermedia* and *P. nigrescens*, therefore it may be possible that the amplification of band II plus additional bands differentiates the PINLOs. Cluster analysis confirms species divisions and it is suggested that such analyses is always undertaken to confirm visual results.

4.18.2.2 Partial sequencing of 16S rRNA gene

PINLOs represent a previously undescribed species (Devine *et al.*, 1997). This analysis of a variable region confirms that PINLOs are not *P. intermedia* (as suggested by the occurrence of bands II and IV) and are also unlikely to represent a subspecies due to the fact that they have the highest level of 16S rRNA sequence similarity to *P. corporis*. Due to the possible identical nature of the 16S rRNA gene sequence in this region, complete 16S rRNA gene sequences are required before further conclusions can be drawn regarding these strains.

4.18.2.3 PCR with *P. intermedia* and *P. nigrescens* specific primers

Although DNA from the three PINLOs was used as a template for both *P. intermedia* and *P. nigrescens* specific primers and HST 1156 was amplified by primer P-nig and RE-TPU1, no conclusions can be drawn from this as *P. intermedia* MH3 and MH15, *P. pallens* AHN 9423, *P. corporis* ATCC 33547, and *P. corporis* A350 were also amplified. This suggests a lack of specificity of this primer above the identification of HST 1156 as *P. nigrescens*, especially when RAPD-PCR and sequencing data are considered.

DISCUSSION

SUMMARY AND FURTHER WORK

4.19 OVERALL SUMMARY OF WORK

The work included in this thesis was split into two broad topic areas. The use of molecular techniques for the differentiation and identification of *P. intermedia* and *P. nigrescens* was examined. The same techniques were used to study and identify clinical *P. intermedia* isolates cultured from plaque of patients with PLS and PINLOs. In the second part a range of identification techniques were used to identify a large number of bacterial isolates cultured from the plaque of patients with PLS which included those identified as *P. intermedia*. In conclusion the major findings of this study are as follows.

4.19.1 RAPD-PCR

1. Condition optimisation and standardisation ($MgCl_2$, primer concentration, annealing temperature, thermal cycler, reagent supplies and DNA extractions) are crucial for RAPD-PCR when using primer L10.
2. The use of template DNA within the range 500 ng to 2.5 ng (per 50 µl of reaction mix) does not significantly alter the banding pattern when using primer L10 for amplification.
3. Extracted DNA is stable and can be stored and used for RAPD-PCR amplification over an extended (15 month) period without significant alterations to the amplification profiles when using primer L10.

4.19.2 SPECIES-SPECIFIC PCR PRIMERS

1. The 16S rRNA gene sequence of *P. intermedia* and *P. nigrescens* was confirmed to be a suitable template for PCR primers.

4.19.3 PARTIAL 16S RRNA GENE SEQUENCING

1. The use of partial 16S rRNA gene sequencing allows the most probable relationship of an unknown clinical isolate from PLS to be determined in relation to known oral species previously characterised in this way and therefore identified.
2. Partial 16S rRNA gene sequencing allows species relationships to be examined as illustrated here for *Prevotella* species.

4.19.3.1 Differentiation and identification of *P. intermedia* from *P. nigrescens*

3. The failure of commercial anaerobe identification kits (Rapid ID 32 A and RapID ANA II system), traditional laboratory test and GLC to differentiate between *P. intermedia* and *P. nigrescens* was confirmed.

4. RAPD-PCR with primer L10 and partial 16S rRNA gene sequencing using PCR primers RE-TPU1 and RE-RTU3 will differentiate members of *P. intermedia* and *P. nigrescens*.
5. RAPD-PCR with primer L10 offers a successful method for the differentiation of *P. intermedia* from *P. nigrescens* by the amplification of marker bands.
6. Cluster analysis of RAPD-PCR amplification profiles obtained with primer L10 results in two distinct clusters corresponding to *P. intermedia* and *P. nigrescens* whilst illustrating strain and species heterogeneity.
7. Partial 16S rRNA gene sequencing allows the most probable relationship of an unknown isolate provisionally identified as *P. intermedia* (on the basis of biochemical tests) to be determined in relation to known strains of *P. intermedia* or *P. nigrescens* and therefore identified.

4.19.4 COMPARISON OF PINLOS TO *P. INTERMEDIA* AND *P. NIGRESCENS*

1. PINLOs are identified as *P. intermedia* using commercial identification kits and GLC.
2. RAPD-PCR with primer L10 shows that PINLOs are distinct from *P. intermedia* or *P. nigrescens*.
3. Visual analysis and cluster analysis of amplification profiles of RAPD-PCR with primer L10 confirms that PINLOs are distinct from *P. corporis*, *P. intermedia*, *P. nigrescens*, or *P. pallens*.
4. Partial 16S rRNA gene sequencing confirms a close relationship of PINLOs to other *Prevotella* species, especially *P. corporis*.
5. PINLOs probably represent at least one previously undescribed species of *Prevotella*.

4.19.5 PAPILLON-LEFÈVRE SYNDROME

1. Commercial identification kits used alone are not sufficient to identify clinical isolates.
2. GLC is valuable for the identification of anaerobic and facultative species.
3. Partial 16S rRNA gene sequences allow most probable relationships of unknown isolates to be determined providing identification.
4. Aerobic species are predominantly *Neisseria*.
5. Facultative anaerobes are predominantly streptococci
6. Anaerobic species include *Prevotella* and *Peptostreptococcus*.
7. Both *P. intermedia* and *P. nigrescens* have been isolated from the oral cavity of patients with PLS.
8. PLS had a microbial aetiology similar to adult periodontitis.

4.20 FURTHER WORK

The research that is included in this thesis could be continued and suggested directions are detailed in the following section.

4.20.1 RAPD-PCR

Further testing of primers 970-11 and US possibly newly synthesised oligonucleotides and further optimisation experiments (e.g. annealing temp) may enable strain typing studies for *P. intermedia* and *P. nigrescens*. The species-specificity of the marker bands requires further investigation due to the occurrence of bands II and IV in PINLO A391 (section 3.8). This would involve examining more strains of *P. intermedia*, *P. nigrescens* and PINLO and may lead to trying alternative random primers. Combining the amplification profiles from 2 or more primers (one of which being L10) could prove useful for species (or strain) differentiation, in the event that bands II and IV are not species-specific. This would also give some statistical significance to the results.

Once the certainty of species marker bands was established, studies involving other *Prevotella* species should be expanded. This would facilitate a control for species marker bands and a study of the relationships between PINLOs and other *Prevotella* species. As the numbers of comparison organisms becomes larger, the researcher is faced with the logistical problem of profile comparison and loading all samples onto a single gel may not be practical. The use of computer measurement should be considered.

4.20.2 *P. INTERMEDIA* AND *P. NIGRESCENS* PRIMERS

An alternative use of the single oligonucleotides is as nucleic acid probes (section 4.15.1) and work could be done to test the feasibility of this (section 4.20.5.1).

The *P .intermedia* and *P. nigrescens* primers designed here require further optimisation, as described in section 4.6.7, this is especially true of the *P. nigrescens* primers.

Further study of 16S rRNA gene sequences of *P. intermedia* and *P. nigrescens* may reveal other potential sequences which could be tested. Ideally, primer pairs that are as closely matched as possible in terms of GC content and T_m should be designed, it is therefore possible that specificity will be increased by designing a specific forward primer rather than using RE-TPU1. Alterations to either primer necessitate repeated optimisation procedures. The *P. intermedia* primers P-int and 1Bi-1 are working satisfactorily, but further specificity studies are required to achieve amplification of all *P. intermedia* strains.

Once a primer pair appeared to work specifically with a limited range of organisms as tested in this study, increased numbers of species would be required as templates.

A suitable primer pair could therefore be exploited for detection and identification of *P. intermedia* and *P. nigrescens* directly from dental plaque and this can be considered the ultimate application for all working primer pairs. Purified DNA extracted from either species would be used as an internal control for clinical studies. This clinical application should be coupled with a quick DNA extraction protocol (Parrish and Greenberg, 1995) to allow rapid results which should be tested thoroughly. In addition, the lowest limit of detection in terms of the number of bacterial cells it represents should be known. The affect of a mixed species plaque sample on the sensitivity and specificity should be determined (Conrads *et al.*, 1997) especially if a clinical application is required.

4.20.3 16S RRNA GENE SEQUENCE ANALYSIS

The use of 16S rRNA gene sequences could be enhanced by the use of alternative primers which amplify a longer length of sequence (Lane, 1991; Weisburg *et al.*, 1991). This would enable more accurate studies of the heterogeneity amongst *P. intermedia* and *P. nigrescens*. For PINLOs and clarifying the identity of clinical isolates, the complete 16S rRNA gene sequence is desirable and necessary if phylogenetic studies of PINLOs are to be undertaken.

4.20.4 EXAMINATION OF PINLOS

A full phylogenetic study of the PINLO strains is required. This would involve a detailed study of growth, carbohydrate fermentation, enzyme activities and mobilities (due to relationship with *P. intermedia* and *P. nigrescens*), antibiotic susceptibilities, GLC of metabolic end-products, cellular fatty acid composition, DNA-DNA hybridization studies and complete 16S rRNA gene sequencing.

4.20.5 IDENTIFICATION OF ISOLATES FROM PLAQUE OF PAPILLON-LEFÈVRE SYNDROME PATIENTS

An alternative identification scheme is provided in section 4.15. The majority of isolates are preserved in FUM (appendix 6.1.5), therefore identifications could be checked if desired. Alternatively, partial 16S rRNA gene sequence analysis could be performed using all isolates.

4.20.5.1 Nucleic acid probes

The use of nucleic acid probes for the detection of specific organisms has become commonplace in the study of periodontal microbiology, offering advantages over other techniques for detecting fastidious organisms (section 1.8.7.2). The sequences used in this study as PCR primers could be tested as probes.

The use of a nucleic acid probes can be broken down into 5 steps as defined by Schleifer *et al.* (1993). (1) design of a single stranded probe complementary to a region of target organism; (2) attaching a label to the probe (3) prepare target nucleic acid, either pure or mixed culture; (4) hybridization in the desired format; (5) detection of the double stranded hybrids. With the exception of step 1 which has been done (see section 4.6.1), and step 3 which is discussed in section 4.2, steps 2 4 and 5 are covered briefly below.

Labelling the probe falls into two categories; radioactive and non-radioactive labels which are safer (Karcher, 1995; Schleifer *et al.*, 1993). These can again be split into two categories direct where the label is bound to the probe, and indirect where an unlabelled molecule is bound to the probe which is in turn detected by a binding molecule (Schleifer *et al.*, 1993). Direct labels include radioisotopes and horseradish-peroxidase (Schleifer *et al.*, 1993) and indirect ones include biotin and Digoxygenin (DIG) which are incorporated into the DNA and detected by streptavidin or anti-DiG antibody respectively and a chromogenic or chemiluminogenic substrate (Karcher, 1995).

There are 4 ways in which hybridization reactions can be conducted; on a solid (filter, slot-, dot-blot) support, in solution, in situ or using southern hybridization (Albanar and Olsen, 1990; Tenover, 1988) and the reader is referred to other texts (Schleifer *et al.*, 1993; Tenover, 1988) for details. The majority of work (Albanar and Olsen, 1990) detecting oral pathogens is done using dot- (Conrads and Brauner, 1995; Conrads *et al.*, 1996; DiRienzo *et al.*, 1991; French *et al.*, 1986; Lotufo *et al.*, 1994; Mättö *et al.*, 1996b) and slot-blot (Lippke *et al.*, 1991) hybridizations. Dot- and slot- blot hybridization (Schleifer *et al.*, 1993) allow pure and mixed cultures to be analysed for the presence of absence of a particular sequence and therefore a particular species (Schleifer *et al.*, 1993). The nucleic acid is applied directly to a membrane in dots manually or through slots in a vacuum chamber (Schleifer *et al.*, 1993). Unbound probe is removed by washing (Engleberg, 1994; Savitt *et al.*, 1990) which is done under high stringency to ensure the specificity of the reaction (Karcher, 1995; Schleifer *et al.*, 1993).

The amount of labelled and bound probe is detected by the addition of chromogenic substrates which can be recorded qualitatively or by ELISA or chemiluminogenic substrates

which are detected on x-ray film (Karcher, 1995). This is done after unbound probe has been removed by washing).

During this study, two potential sources of nucleic acid probe have been revealed, exploitation of either would necessitate sensitivity and specificity studies.

4.20.5.1.1 Species-specific bands amplified by RAPD-PCR

Species-specific bands amplified by RAPD-PCR could be used to produce species-specific probes. These have a high specificity due to the number of RAPD-PCR experiments which need to be conducted to determine the presence of a species-specific band (Ménard *et al.*, 1994). This has been done for *Porphyromonas gingivalis* (Ménard *et al.*, 1994), *Leptospira* sp., (Letocart *et al.*, 1997) and *Mycobacterium bovis* (Rodriguez *et al.*, 1995). The desired band is excised from the electrophoresis gel and purified using published methods (Karcher, 1995; Sambrook *et al.*, 1987). The sequence can be amplified by PCR using the same random primer (Letocart *et al.*, 1997), observed by gel electrophoresis to ascertain purity (Letocart *et al.*, 1997), labelled as described above (section 4.20.5.1) and used for dot-blot hybridization (Letocart *et al.*, 1997; Schleifer *et al.*, 1993) or southern blot hybridization (Rodriguez *et al.*, 1995).

4.20.5.1.2 Species-specific primers

The sequences that were designed as PCR primers could be tested as species-specific nucleic acid probes, as 1Bi-1 and 2Bi-1 have been (Mättö *et al.*, 1996b; Shah *et al.*, 1995). The sequences could be labelled as described above (section 4.20.5.1) and used for dot-blot hybridization (section 4.20.5.1). Dot-blot hybridization could be used with a clinical perspective for qualitative analysis (Schleifer *et al.*, 1993) of plaque samples to determine whether or not *P. intermedia* or *P. nigrescens* are present. The use of colony-lifts (hybridization) has also been reported (Haffajee *et al.*, 1992), thus coupling species detection with total viable counts from clinical samples (see Schleifer *et al.*, 1993 for description of colony-hybridization) which could be considered as an alternative method. Only minimal treatment of a subgingival plaque sample would be necessary before probe detection (Schleifer *et al.*, 1993). A combination of detergent to lyse bacterial cells and boiling at a high pH to denature DNA has been reported (Söder *et al.*, 1993).

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6. APPENDICES

6.1 GROWTH MEDIUM

6.1.1 STERILISATION OF GROWTH MEDIUM

All media were sterilised by autoclave at 121°C for 15 min (15 lb/in^2, 1.05 bar; Cowan and Steel, 1993). It was then allowed to cool to 50°C before pouring into sterile petri dishes, sterile bottles or the addition of blood to make blood agar (see section 6.1.2 below).

6.1.2 BLOOD AGAR PLATES

The agar base was cooled to approximately 50°C in a water bath and 5% (v/v) defibrinated horse blood was added aseptically. The components were mixed avoiding air bubbles and poured into sterile plastic petri dishes. These were allowed to set and were pre-reduced in an anaerobic environment overnight before use.

6.1.3 SOLID MEDIUM

6.1.3.1 Fastidious Anaerobe Agar (FAA)

FAA was used to make blood agar plates. The powder (46 g/l) was dissolved in distilled water and sterilised as above (section 6.1.1), 5% (v/v) defibrinated horse blood was added before pouring into petri dishes.

The commercial powder contains peptone mixture (15.0 g/l); yeast extract (10.0 g/l); sodium thiolycollate (0.5 g/l); sodium chloride (2.5 g/l); L-cysteine HCl (0.5 g/l); resazurin (0.001 g/l); sodium bicarbonate (0.4 g/l); haemin (0.005 g/l) and vitamin K (0.0005 g/l).

6.1.3.2 Columbia Blood Agar

Columbia agar base was used to make Columbia blood agar plates. The powder (41 g/l) was dissolved in distilled water and sterilised as above (section 6.1.1), 5% (v/v) defibrinated horse blood was added before pouring into petri dishes.

The components are as follows: Columbia peptone mixture (23.0 g/l); corn starch (1.0 g/l); sodium chloride (5.0 g/l) and agar number 2 (12.0 g/l).

6.1.3.3 Tryptone Yeast Cysteine

TYC agar plates were prepared by dissolving 98 g/l of commercial powder and sterilising (section 6.1.1). Blood was not added.

The components are as follows: tryptone (15.0 g/l); yeast extract (5.0 g/l); L-cysteine (0.2 g/l); sodium sulphate (0.1 g/l); sodium chloride (1.0 g/l); disodium phosphate anhydrous

(0.8 g/l); sodium bicarbonate (2.0 g/l); sodium acetate anhydrous (12.0 g/l); sucrose (50.0 g/l) and agar number 2 (12.0 g/l).

6.1.3.4 Egg yolk agar

FAA plates (1.3.1) were prepared as described in section 6.1.2. No blood was added. FAA was allowed to cool to approximately 50°C in a water bath and 5% (v/v) egg yolk emulsion was added aseptically. The components were mixed avoiding air bubbles and poured into sterile plastic petri dishes. These were allowed to set.

6.1.4 LIQUID MEDIUM

All types of liquid medium was treated in the same way. The commercial powder was dissolved in distilled water and autoclaved at 121°C for 15 min (15 lb/in^2, 1.05 bar; Cowan and Steel, 1993). It was allowed to cool to a comfortable (hand-held) temperature before aliquotting into sterile containers (bijoux bottles or universal bottles).

6.1.4.1 Fastidious Anaerobe Broth (FAB)

FAB commercial powder (29.7 g/l) was dissolved in distilled water and sterilised.

The components are as follows: peptone mixture (15.0 g/l); yeast extract (10.0 g/l); sodium thioglycollate (0.5 g/l); sodium chloride (2.5 g/l); agar number 1 (0.75 g/l); l-cysteine HCl (0.5 g/l); resazurin (0.001 g/l); sodium bicarbonate (0.4 g/l); haemin (0.005 g/l) and vitamin K (0.0005 g/l).

6.1.4.2 Brain Heart Infusion Broth (BHI broth)

BHI broth commercial powder (37 g/l) was dissolved in distilled water and sterilised.

The components are as follows: BHI solids (17.5 g/l); tryptose (10.0 g/l); glucose (2.0 g/l); sodium chloride (5.0 g/l) and disodium hydrogen phosphate (2.5 g/l).

6.1.4.3 Gas Liquid Chromatography Broth (GLC broth)

The commercial powder (28.9 g/l) was dissolved in distilled water.

The components are as follows: peptone mixture (15.0 g/l); yeast extract (10.0 g/l); sodium thioglycollate (0.5 g/l); sodium chloride (2.5 g/l); cysteine hydrochloride (0.5 g/l); haemin (0.005 g/l); vitamin K (0.0005 g/l) and sodium bicarbonate (0.4 g/l).

6.1.5 LONG TERM STORAGE MEDIUM (MODIFIED FLUID MEDIUM; FUM)

The following components of FUM (per litre of distilled water) were dissolved, mixed and sterilised at 121°C for 15 min (15 lb/in^2, 1.05 bar; Cowan and Steel, 1993). FUM (fluid medium) was first described by Loesche *et al.* (1972) as MM10 medium and modified by Gmür and Guggenheim (1983).

Tryptone (10 g); yeast extract (5 g); cysteine hydrochloride (0.5 g); sodium chloride NaCl (0.9 g); sodium carbonate Na_2CO_3 (0.5 g); potassium nitrate KNO_3 (1 g); potassium phosphate monobasic KH_2PO_4 (0.45 g); potassium phosphate dibasic trihydrate $K_2HPO_4.3H_2O$ (0.45 g); ammonium sulphate $(NH_4)_2SO_4$ (0.9 g); 1 ml equine haemin (0.02 g in 10 ml 1 M NaOH) and 1 ml menadione / vitamin K (0.01 g in 10 ml absolute alcohol). The pH was adjusted to 7.1. Using aseptic technique, 9.2 ml was aliquotted into sterile universals. The medium can be stored at 4°C in this point. Prior to inoculation, 100 µl sterile magnesium sulphate (x100 concentration); 200 µl sterile glucose (x50 concentration) and 500 µl horse serum are added to the medium. After the addition of horse serum, the medium could not be resterilised.

6.2 COMMERCIAL KITS

See appendix 6.14 and 6.15 for details concerning commercial identification kits

6.2.1 PUREGENE DNA ISOLATION KIT FOR ISOLATING DNA FROM GRAM-POSITIVE BACTERIA

Each kit contains; cell lysis solution (1.5 ml), protein precipitation solution (0.5 ml), DNA hydration solution (0.5 ml), RNase A solution (7.5 ml), cell suspension solution (1.5 ml) and lytic enzyme solution (7.5 ml). RNase A solution was stored at 4°C and all other reagents at room temperature. Additional reagents required but not supplied *iso*-propanol (propan-2-ol) and ethanol (70%). In addition to solutions and chemicals, a microfuge, micro (Gilson) pipettes, sterile 1.5 ml microfuge tubes and sterile disposable plastic pipette tips were required.

The protocol is described fully in the manufacturer's instructions and is summarised briefly below. Cells were harvested from 1 ml bacterial culture in BHI and resuspended in cell suspension solution. Lytic enzyme solution was added to digest microbial cell walls and cell lysis solution to initiate cell lysis. RNA was removed by RNase A treatment, which was followed by protein precipitation. DNA was precipitated using *iso*-propanol and ethanol and rehydrated (DNA hydration solution). Extracted DNA was stored at 4°C.

6.2.2 QIAQUICK PCR PURIFICATION KIT (QIAGEN)

This kit was used to purify PCR products for sequence analysis.

The kit contains; buffer PB, buffer PE (components not given) and buffer EB (10 mM Tris-chloride, pH 8.5), 2 ml collection tubes and QIAquick spin columns.

In addition to these buffers, a microfuge, micro (Gilson) pipettes and sterile disposable plastic pipette tips were required.

The protocol is described fully in the manufacturer's instructions and is summarised briefly below. Five volumes (250 µl) of buffer PB was added to the PCR reaction, removal of mineral oil was not necessary. This was transferred to a QIAquick spin column in a collection tube and centrifuged for 1 min. Buffer PE (0.75 ml) was added to the column and centrifuged for 1 min and the tube emptied before centrifuging for an additional 1 min. The collection tube was emptied between each centrifugation. The DNA was then eluted into a clean tube by adding 50 µl of buffer EB and centrifuging for 1 min.

6.3 RAPD-PCR - ABSENCE OF AMPLIFICATION PRODUCTS

In instances were no PCR amplification products were seen, three parameters were explored whilst keeping other factors constant.

6.3.1 DNA

The RAPD-PCR reaction in question was repeated (see materials and methods section 2.5) using DNA from an alternative extraction and prepared in a fresh dilution.

6.3.2 ACTIVITY OF *TAQ* POLYMERASE

DNA which had been successfully amplified in the past was subjected to RAPD-PCR (see materials and methods section 2.5) using current and fresh *Taq* in parallel reactions. Amplification products were compared to show that the activity of *Taq* was normal

6.3.3 OPERATOR ERROR

The PCR reaction in question was carried out (see materials and methods section 2.5), by 2 persons in parallel. Resultant profiles were checked for identity.

6.4 RAPD-PCR BAND SIZES

The approximate sizes of the bands to be scored were determined. This was done by constructing a reference curve displaying the fragment size for the molecular weight marker Lambda *Pst*1 (Sigma) plotted versus the distance moved by the fragment. Lambda *Pst*1 contains fragments of sizes 11497 bp to 15 bp. The smallest fragment which could be routinely seen was the doublet comprising two fragments, 514 bp and 468 bp. The distance moved by fragments could then be used to determine the approximate size (bp).

Table 6.1 Size (bp) of bands that were scored following amplification of strains of *Prevotella* species with primer L10

A) Known strains of *P. intermedia* and *P. nigrescens*. Each size (bp) is represented by a coloured dot in figure 6.1.

B) Known strains of *P. intermedia* and *P. nigrescens* plus clinical isolates A-D. Labels a-c correspond to figure 3.8

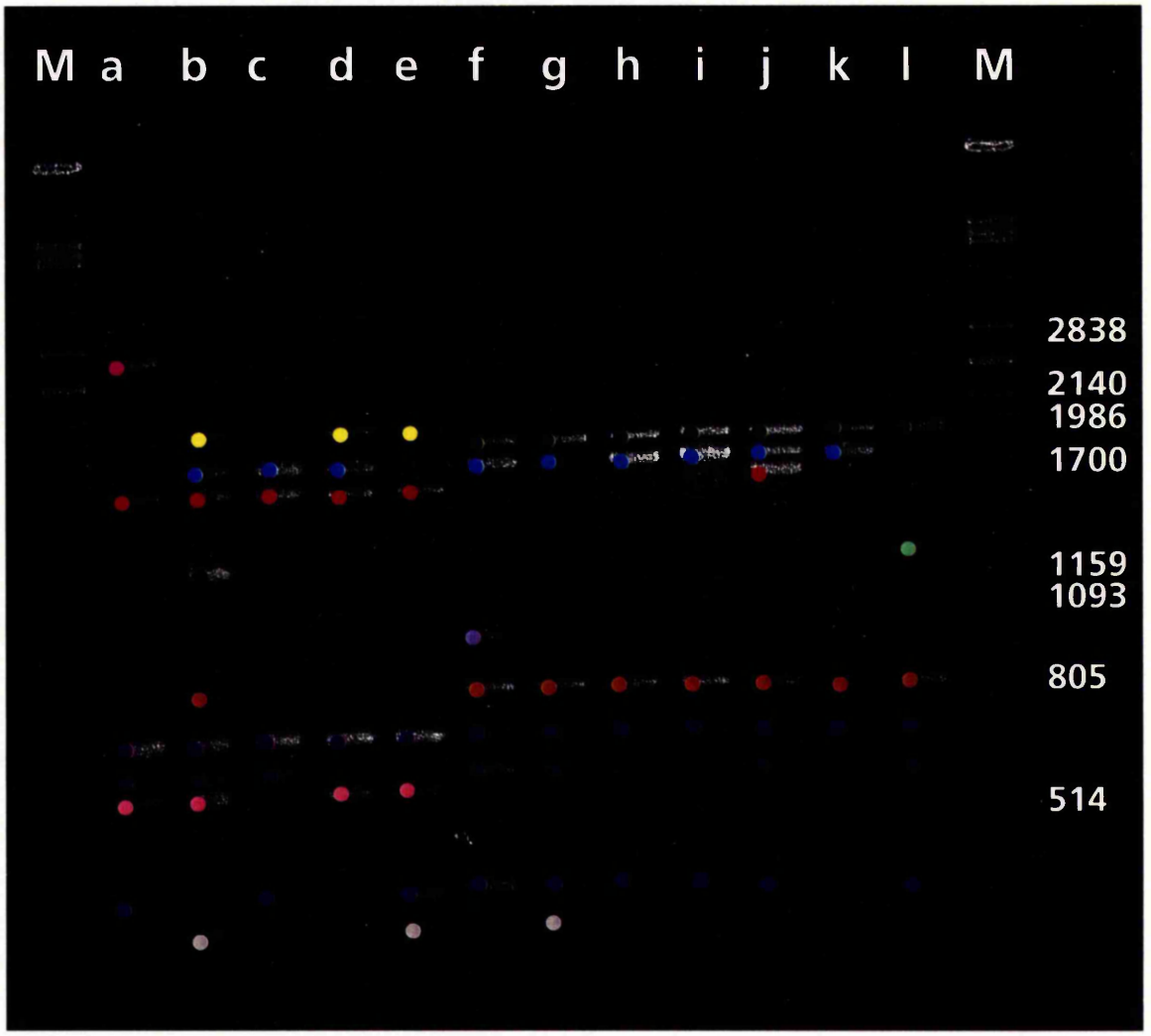
C) Known strains of *P. intermedia* and *P. nigrescens*, clinical isolate A-D, *P. corporis*, *P. intermedia*, *P. nigrescens*, *P. pallens* and PINLOs. Labels a-c correspond to figure 3.10.

(A) SIZE IN
2600
2000
1900
1750
1650
1250
1200
975
875
805
600
575
514
400
350

(B) SIZE IN
2600
2000
1900
1750
1650
1250
1200
a - 1180
b - 1050
975
875
805
600
575
514
c - 480
400
350

(C) SIZE IN	
a - 2140	805
2000	780
1900	c - 600
b - 1750	590
1650	575
1600	514
1500	500
1450	495
1330	480
1250	400
1200	350
1159	< 350
1090	< 350
930	< 350
900	< 350
875	< 350

Figure 6.1 RAPD-PCR of *P. intermedia* and *P. nigrescens*; bands scored for analysis



KEY:

Lanes a-e, *P. intermedia* strains. a, *P. intermedia* MH3; b, *P. intermedia* MH6; c, *P. intermedia* MH12; d, *P. intermedia* MH15; e, *P. intermedia* ATCC 25611

Lanes f-l, *P. nigrescens* strains. f, *P. nigrescens* MH1; g, *P. nigrescens* MH2; h, *P. nigrescens* MH4; i, *P. nigrescens* MH5; j, *P. nigrescens* LM94; k, *P. nigrescens* ATCC 25261; l, *P. nigrescens* ATCC 33563

M, molecular weight marker (λ *Pst* I digest, fragment sizes in bp)

FOOTNOTE TO FIGURE 6.1:

1. Figure 6.1 is the same as figure 3.6
2. Each coloured circle represents a band of the same size (see table 6.1. (a)).
3. Any visible bands not scored in this figure were inconsistently amplified

6.5 INTERNATIONAL BASE CODES

N; A, C, T and G

R; A and g

Y; C and T

M; A and C

K; T and g

S; c and g

W; A and T

M; A, T and c

B; T, c and g

D; A, T and g

V; A, c and g

6.6 EMBL INFORMATION FOR THE BACTERIAL SPECIES USED FOR 16S RDNA SEQUENCE ALIGNMENT

The organisms in this list were not sequenced during this study but sequences were aligned and used for distance calculations and dendrogram construction in comparison with PINLOs (see section 2.6.8.2).

Identification and accession numbers refer to the EMBL database.

Table 6.2 ***Prevotella*** **species used for comparison of 16S rDNA sequences of *P. intermedia, P. nigrescens* and / or PINLO strains.**

	ID	Accession No
P. corporis ATCC 33547	PVORR16SA	L16465
P. denticola ATCC 33185	PVORR16SB	L16466
P. denticola ATCC 35308	PVORR16SC	L16467
P. intermedia ATCC 25611	PVORR16SD	L16468
P. intermedia ATCC 25611	PIRRNA	X73965
P. nigrescens ATCC 25261	PVORR16SO	L16479
P. nigrescens ATCC 33563	PVORR16SG	L16471
P. nigrescens NCTC 9336	PNRRNA	X73963
P. pallens strain 10371	PP16S1037	Y13105
P. pallens strain 8792	PP16S8792	Y13107
P. pallens strain 9423	PP16S9423	713106

6.7 PARTIAL 16S rDNA SEQUENCES – *PREVOTELLA SPECIES* AND PINLO STRAINS

6.7.1 *P. INTERMEDIA*

*Pi*6, *P. intermedia* MH6: Length: 457

*Pi*12, *P. intermedia* MH12: Length: 470

*Pi*3, *P. intermedia* MH3: Length: 440

*Pi*15, *P. intermedia* MH15: Length: 438

```
      1                                                   50
 Pi6  ..TAGGCTTA ACACATGCAA GTCGAGGGGA AACGGCATTA TGTGCTTGCA
Pi12  TATAGGCTTA ACACATGCAA GTCGAGGGGA AMCGGCATTA TGTGCTTGCA
 Pi3  ..TAGGCTTA ACACATGCAA GTCGAGGGGA AACGGCATTA TGTGCTTGCA
Pi15  .......... .......... .....GGGGA AACGGCATTA TGTGCTTGCA

      51                                                  100
 Pi6  CATTCTGGAC GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTTC
Pi12  CATTCTGGAC GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTTC
 Pi3  CATTTTGGAC GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTTC
Pi15  CATTCTGGAC GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTTC

      101                                                 150
 Pi6  CCTCCACTCG GGGATACCCC GTTGAAAGAC GGCCTAATAC CCGATGTTGT
Pi12  CCTCCACTCG GGGATACCCC GTTGAAAGAC GGCCTAATAC CCGATGTTGT
 Pi3  CCTCCACTCG GGGATACCCC GTTGAAAGAC GGCCTAATAC CCGATGTTGT
Pi15  CCTCCACTCG GGGATACCCC GTTGAAAGGC GGCCTAATAC CCGATGTTGT

      151                                                 200
 Pi6  CCACATATGG CATCTGACGT GGACCAAAGA TTCATCGGTG GAGGATGGGG
Pi12  CCACATATGG CATCTGACGT GGACCAAAGA TTCATCGGTG GAGGATGGGG
 Pi3  CCACATATGG CATCTGACGT GGACCAAAGA TTCATCGGTG GAGGATGGGG
Pi15  CCACATATGG CATCTGACGT GGACCAAAGA TTCATCGGTG GAGGATGGGG

      201                                                 250
 Pi6  ATGCGTCTGA TTAGCTTGTT GGTGCGGGTA ACGGCCCACC AAGGCAACGA
Pi12  ATGCGTCTGA TTAGCTTGTT GGTGCGGGTA ACGGCCCACC AAGGCTACGA
 Pi3  ATGCGTCTGA TTAGCTTGTT GGTGCGGGTA ACGGCCCACC AAGGCGACGA
Pi15  ATGCGTCTGA TTAGCTTGTT GGTGCGGGTA ACGGCCCACC AAGGCGACGA

      251                                                 300
 Pi6  TCAGTAGGGG TTCTGAGAGG AAGGTCCCCC ACATTGGAAC TGAGACACGG
Pi12  TCAGTAGGGG TTCTGAGAGG AAGGTCCCCC ACATTGGAAC TGAGACACGG
 Pi3  TCAGTAGGGG TTCTGAGAGG AAGGTCCCCC ACATTGGAAC TGAGACACGG
Pi15  TCAGTAGGGG TTCTGAGAGG AAGGTCCCCC ACATTGGAAC TGAGACACGG

      301                                                 350
 Pi6  TCCAAACTCC TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGACGTA
Pi12  TCCAAACTCC TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGACGTA
 Pi3  TCCAAACTCC TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGACGTA
Pi15  TCCAAACTCC TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGACGTA
```

```
      351                                                        400
 Pi6  AGTCTGAACC AGCCAAGTAG CGTGCAGGAT TGACGGCCCT ATGGGTTGTA
Pi12  AGTCTGAACC AGCCAAGTAG CGTGCAGGAT TGACGGCCCT ATGGGTTGTA
 Pi3  AGTCTGAACC AGCCAAGTAG CGTGCAGGAT TGACGGCCCT ATGGGTTGTA
Pi15  AGTCTGAACC AGCCAAGTAG CGTGCAGGAT TGACGGCCCT ATGGGTTGTA

      401                                                        450
 Pi6  AACTGCTTTT GTTGGGGAGT AAAGTGAGGC ACGCGTGCCT TTTTGCATTT
Pi12  AACTGCTTTT GTTGGGGAGT AAAGTGAGGC ACGCGTGCCT TTTTGCATTT
 Pi3  AACTGCTTTT GTTGGGGAGT AAAGTGAGGC ACGTGTGCCT TT........
Pi15  AACTGCTTTT GTTGGGGAGT AAAGTGAGGC ACGCGTGCCT TTTTGCATTT

      451               470
 Pi6  ACCCTTCGA. ..........
Pi12  ACCCTTCGAA TAAGGACCGG
 Pi3  .......... ..........
Pi15  ACCCTTCGAA TAA.......
```

6.7.2 *P. NIGRESCENS*

*Pn*5, *P. nigrescens* MH5: Length: 426

*Pn*2, *P. nigrescens* MH2: Length: 400

*Pn*1, *P. nigrescens* MH1: Length: 409

*Pn*94, *P. nigrescens* LM94: Length: 470

*Pn*4, *P. nigrescens* MH4: Length: 458

```
      1                                                          50
Pn5   .......... .......... .....GTGGG GAAACGGCAT TATGTGCTTG
Pn94  GCTACAGGCT TAACACATGC AAGTCGTGGG GAAACGGCAT TATGTGCTTG
Pn2   .......... .......... .......... ........AT TATGTGCTTG
Pn4   .......... ..ACACATGC AAGTCGTGGG GAAACGGCAT TATGTGCTTG
Pn1   .......... .......... .......... .........T TATGTGCTTG

      51                                                         100
Pn5   CACATTCTGG ACGTCGACCG GCGCACGGGT GAGTATCGCG TATCCAACCT
Pn94  CACATTCTGG ACGTCGACCG GCGCACGGGT GAGTATCGSG TATCCAACCT
Pn2   CACATTCTGG ACGTCGACCG GCGCACGGGT GAGTATCGCG TATCCAACCT
Pn4   CACATTCTGG ACGTCGACCG GCGCACGGGT GAGTATCGCG TATCCAACCT
Pn1   CACATTCTGG ACGTCGACCG GCGCACGGGT RAGTATCGCG TATCCAACCT

      101                                                        150
Pn5   GCCCCTTACT TGGGGATACC CCGTTGAAAG ACGGCCTAAT ACCCGATGTG
Pn94  GCCCCTTAMT TGGGGATACC CCGTTGAAAG ACGGCCTAAT ACCCGATGTG
Pn2   GCCCCTTACT TGGGGATACC CCGTTGAAAG ACGGCCTAAT ACCCGATGTG
Pn4   GCCCCTTACT TGGGGATACC CCGTTGAAAG ACGGCCTAAT ACCCGATGTG
Pn1   GCCCCTTACT TGGGGATACC CCGTTGAAAG ACGGCCTAAT ACCCGATGTG

      151                                                        200
Pn5   TTTCATTGAC GGCATCCGAT ATGAAACAAA GGTTTTTCCG GTAAGGGATG
Pn94  TTTCATTGAC GGCATCCGAT ATGAAACAAA GGTTTTYCCG GTAAGGGATG
Pn2   TTTCATTGAC GGCATCCGAT ATGAAACAAA GG.TTTTCCG GTAAGGGATG
Pn4   TTTCATTGAC GGCATCCGAT ATGAARCRAA GG.TTTTCCG GTAARGGATG
Pn1   TTTCATTGAC GGCATCCGAT ATGAAACAAA GG.TTTTCCG GTAAGGGATG
```

```
     201                                                        250
Pn5  GGGATSCKTC TGATTASCTT GTTGGCGGGG CAACGGCCCA CCAAGGCRAC
Pn94 GGGATSCKTC TgATTASCTT gTTGGCGGGG CAACGGCCCA CCAAGGCRAC
Pn2  GGGATGCGTC TGATTAGCTT GTTGGCGGGG CAACGGCCCA CCAAGGCGAC
Pn4  GGGATGCGTC TGATTARCTT GTTGGCGGGG CAACGGCCCA CCAAGGCGAC
Pn1  GGGATGCGTC TGATTAGCTT GTTGGCGGGG CAACGGCCCA CCAAGGCGAC

     251                                                        300
Pn5  RATCARTAGG GGTTCTGARA GGAAGGTCCC CCACATTGGA ACTGARACAC
Pn94 RATCAKTAGG GGTTCTGAgA GGAAGGTCCC CCACATTGGA ACTGARACAC
Pn2  GATCAGTAGG GGTTCTGAGA GGAAGGTCCC CCACATTGGA ACTGAGACAC
Pn4  GATCAgTAGG GGTTCTGAgA gGAAGGTCCC CCACATTGGA ACTGAGACAC
Pn1  GATCAGTAGG GGTTCTGAGA GGAAGGTCCC CCACATTGGA ACTGAGACAC

     301                                                        350
Pn5  GGTCCAAACT CCTACGGGAG GCASCAKTGA GGAATATTGG TCAATGGACG
Pn94 GGTCCAAACT CCTACGGGAG GCAGCAKTGA GGAATATTGG TCAATGGACg
Pn2  GGTCCAAACT CCTACGGGAG GCAGCAGTGA GGAATATTGG TCAATGGACG
Pn4  GGTCCAAACT CCTACGGGAG GCAGCAGTGA gGAATATTGG TCAATGGACG
Pn1  GGTCCAAACT CCTACGGGAG GCAGCAGTGA GGAATATTGG TCAATGGACG

     351                                                        400
Pn5  CRAGTCTGAA CCASCCAAGT ASCGTKCAGG ATRACGGCCC TATGGGTTGT
Pn94 CAAgTCTGAA CCASCCAAgT ASCgTGCAGG ATRACGGCCC TATGGGTTGT
Pn2  CAAGTCTGAA CCAgCCAAGT AGCGTGCAGG ATGACGGCCC TATGGGTTGT
Pn4  CAAGTCTGAA CCAgCCAAGT AKCGTGCAgG ATGACGGCCC TATGGGTTGT
Pn1  CAAGTCTGAA CCAGCCAAGT AGCGTGCAGG ATGACGGCCC TATGGGTTGT

     401                                                        450
Pn5  AAACTGCTTT TATGTGGGAA TAAATTGGCG SACGTGTGCS CCATTGCATG
Pn94 AAACTGCTTT TATKTGGGAA TAAAKTGGCG CACgTGTGCG CCATTGCATg
Pn2  AAACTGCTTT TATGTGGGAA TAAAGTGGCG CACGTGTGC. ..........
Pn4  AAACTGCTTT TATGTGGGAA TAAAGTGGCG CACGTGTGCG CCATTGCATG
Pn1  AAACTGCTTT TATGTGGGAA TAAATTGGCG CACGTGTGCG CCATTGCAT.

     451                 471
Pn5  T......... .......... .
Pn94 TACMTCATGA ATAAGGACCG .
Pn2  .......... .......... .
Pn4  TACMTCATGA ATAAGGACCG G
Pn1  .......... .......... .
```

6.7.3 GRAM-NEGATIVE BLACK-PIGMENTING ANAEROBIC CLINICAL ISOLATES

PLS **1** *2B*; A; *P. nigrescens*: Length: 452 PLS **2** *3A*; B; *P. intermedia*: Length: 399

PLS **5** *1D*; C; *P. intermedia*: Length: 439 PLS **3** *2A*; D; *P. nigrescens*: Length: 418

```
     1                                                          50
12B  CAGGCTTAAC ACATGCAAGT CGTSSSSAAA CGGCATTATG TGCTTGCACA
32A  .......... .......... .....GGAAA CGGCATTATG TGCTTGCACA
23A  .......... .......... .......... .....TTATG TGCTTGCACA
51D  .......... .......... .......AAA CGGCATTATG TGCTTGCACA
```

```
     51                                                  100
12B  TTCTGGACGT CGACCGGCGC ACGGGTGAGT ATCGCGTATC CAACCTGCCC
32A  TTCTGGACGT CGACCGGCGC aCgGGTGAGT ATCGCGTRTC CAACCTGCCC
23A  TTYTGGACGK CGACCGGSGC aCGggTGAGT ATCGCGTAKC CAACCTTCCC
51D  TTCTGGACGT CGACCGGCGC ACGGGTGAgT ATCGCGTATC CAACCTTCCC

     101                                                 150
12B  CTTACTTGGG GATACCCCGT TGAAAGACGG CCTAATACCC GATGTGTTTC
32A  CTTACTTGGG GATACCCCGT TGAAAGACGG CCTAATACCC GATGTGTTTC
23A  TCCACTcgGG GATACCCCGT TGAAAGACGG cCTAATACCC GATGKTGTCC
51D  TCCACTCGGG GATACCCCGT TGAAAgACGG CCTAATACCC GATGTTGTCC

     151                                                 200
12B  ATTGACGGCA TCCGATATGA AACAAAGGTT TTCCGGTAAG GGATGGGGAT
32A  ATTGACGGCA TCCGATRTGA AACAaAGGTT TTCCGGTaAG GGATGGGGAT
23A  ACATRTGGCA TCTGASGKGG ACSAAAGATT CATCGGtGSA GGATGGGGAT
51D  ACATATGGCA TCTGACGTGG ACCAAAgAWT CATCGGTGGA gGaTGGGGaT

     201                                                 250
12B  GCGTCTGATT AGCTAGTTGG CG.GGGCAAC GGCCCACCAA GGCGACGATC
32A  GCgTCTGATT AGCTAGTTGG CG.GGGCAAC GGCCCACCAA GGCGACGATC
23A  GCGtSYGRTT AGCTKGTTGG TGCGGGTAAS gGCCCACCAA GGCGACGATC
51D  GCGtCTGaTT AGCTTGTTGG TGCGGGtAAC GGCCCACCAA GgCTACgatC

     251                                                 300
12B  AGTAGGGGTT CTGAGAGGAA GGTCCCCCAC ATTGGAACTG AGACACGGTC
32A  AGTAGGGGTT CTGAGAGGAA GGTCCCCCAC ATTGGAACTG AGACACGGTC
23A  AGTAGGGGTT CTGAGAGGAA GGTcCCCCAC RTTGGAACTG AGACACGGTc
51D  AGTAGGGGTt cTGAgAGgAR ggtCCCCCAC ATTGGAACTG AGAcACGGTC

     301                                                 350
12B  CAAACTCCTA CGGGAGGCAG CAGTGAGGAA TATTGGTCAA TGGACGCAAG
32A  CAAACTCCTA CGGGAGGCAG CAGTGAGGAA TATTGGTCAA TGGACGCAAG
23A  cAAACTCCTA CGGGAGGCAG CAGTGAGGAA TATTGGTCAA TGGACGGAAG
51D  CAAACTCCTA CGGGRRGCAG CAGTGAGGaA TATTGGTCAA TGGACGKAAG

     351                                                 400
12B  TCTGAACCAG CCAAGTAGCG TGCAGGA.TG ACGGCCCTAT GGGTTGTAAA
32A  TCTGAACCAG CCAAGTAGCG TGCAGGA.TG ACGGCCCTAT GGGTTGTAAA
23A  TCTGAACCAG CCAAGTAGCG TGCAGGATTG ACGGCCCTAT GGGTTGTAAA
51D  YCTGAACCAG CCAAGTAGCG TGCAGGAWTG ACGGCCCTAT GGGTTGTAAA

     401                                                 450
12B  CTGCTTTTAT GTGGGAATAA AGTGGCGCAC GTGTGCGCCA TTGCATGTAC
32A  CTGCTTTTAT GTGGGAATAA AGTGGCGCAC GTGTGCGCCA TTGCA.....
23A  CTGCTTTTGT TGGGGAGTAA AGTGAGGCAC GCGT...... ..........
51D  CTGCTTTTGT TGGGGAKTAA AGTGAGGCAC GTgTgCCYYT TTGCATTTAC

     451          466
12B  CTCA..... ......
32A  .......... ......
23A  .......... ......
51D  CCTTCGAATA AgGACC
```

6.7.4 PINLO STRAINS

HST 1156: Length: 481 HST 2160: Length: 458 A391: Length: 460

```
      1                                                        50
1156  ...CATGCAA GTCGAGGGGA AACGGCATTA AGTGCTTGCA CTTTTTGGAC
2160  ...CATGCAA GTCGAGGGGA AACSGCATTA AGTGCTTGCA CTTTTTGGAC
A391  ACACaTgCAA GTCGAGGGGA AACGGCWYYA AKTGCTTGCA CTTTTTGGAC

      51                                                      100
1156  GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTGC CCATTACTTG
2160  GTCGACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTGC CCATTACTTG
A391  gTCKACCGGC GCACGGGTGA GTATCGCGTA TCCAACCTGC CCATTACTTG

      101                                                     150
1156  GGGATAACCC GTTGAAAGAC GGACTAATAC CCGATGCAGT CCATTGAAGA
2160  GGGATAACCC GTTGAAAGAC GGACTAATAC CCGATGCAGT CCATTGAAGA
A391  GGGATAACCC GTTGAAARAC GGACTAATAC CCGATGCAGT CCATTGAAGA

      151                                                     200
1156  CATCTGAAGC GGACGAAAGA TTTTATCGGT ATTGGATGGG GATGCGTCCG
2160  CATCTGAAGC GGACGAAAGA TTTTATCGGT ATTGGATGGG GATGCGTCCG
A391  CATCTGAAGC GGACRAAAGA TTTTATCGGT ATTGGATGGG GATGCGTCCG

      201                                                     250
1156  ATTAGCTTGT TGGCGGGGTA ACGGCCCACC AAGGCATCGA TCGGTAGGGG
2160  ATTAGCTTGT TGGCGGGGTA ACGGCCCACC AAGGCATSSA TCGGTAGGGG
A391  ATTAGCTTGT TGGCGGGGTA ACGGCCCACC AAGGCATCGA TCGGTAGGGG

      251                                                     300
1156  TTCTGAGAGG AAGGTCCCCC ACATAGGAAC TGAGACACGG TCCTAACTCC
2160  TTCTGAGAGG AAGGTCCCCC ACATAGGAAC TGAGACACGG TCCTAACTCC
A391  TTCTGASAGG AASGTCCCCC ACATAGGAAC TGAGACACGG TCCTAACTCC

      301                                                     350
1156  TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGGCGGT AGCCTGAACC
2160  TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGGCGGT AGCCTGAACC
A391  TACGGGAGGC AGCAGTGAGG AATATTGGTC AATGGGCGGT AGCCTGAACC

      351                                                     400
1156  AGCCAAGTAG CGTGCAGGAT GACGGCCCTA TGGGTTGTAA ACTGCTTTTA
2160  AGCCAAGTAG CGTGCAGGAT GACGGCCCTA TGGGTTGTAA ACTGCTTTTA
A391  AGCCAAGTAK CGTGCAGGAT GACGGCCCTA TGGGTTGTAA ACTGCTTTTA

      401                                                     450
1156  TGCGGGGATA AGTCACTCA CGTGTGAGTG TTTGCAGTTA CCGCATGAAT
2160  TGCGGGGATA AGTCACTCA CGTGTGAGTG TTTGCAGTTA CCGCATGAAT
A391  TGCGGGGATA AGTCACTCA CGTGTGAGTG TTTGCAGTTA CCGCATGAAT

      451                                  484
1156  AAGGACCGGC TAATTCCGTG CCAGCCGCCG CGGT
2160  AAGGACCGGC T......... .......... ....
A391  AAGACCGGCT .......... .......... ....
```

6.8 MANIPULATION OF SEQUENCE INFORMATION

6.8.1 ALL ISOLATES

1. Obtain sequences (see section 2.6)
2. Edit and check sequences obtained (see section 2.6.8) by forward and reverse primers using GAP function
3. FASTA search to provide most likely relationship

6.8.2 *P. INTERMEDIA* AND *P. NIGRESCENS* ISOLATES

1. As above
2. As above (sequences in appendix 6.6.1 and 6.6.3)
3. As above
4. Alignment of *P. intermedia* and *P. nigrescens* sequences with other *P. intermedia and P. nigrescens.*
5. Determination of consensus sequence
6. Removal of uncertain regions of sequence and gaps between alignments
7. Calculation of DISTANCES between nucleic acid sequences
8. Construction of a dendrogram representing the distances between nucleic acid sequences and demonstrating relationship amongst *P. intermedia* and *P. nigrescens* isolates.

6.8.3 PINLO STRAINS

1. As above
2. As above (sequences in appendix 6.6.4)
3. As above
4. Alignment of PINLO sequences with other members of *Prevotella* genus; *P. denticola, P. intermedia, P. melaninogenica, P. nigrescens, P. loescheii, P. pallens.*
5. As above
6. As above
7. As above
8. Construction of a dendrogram representing the distances between nucleic acid sequences and demonstrating relationship between PINLOs and other *Prevotella.*

The following pages contain examples of steps 2, 3, 6, 7 and 8 using PINLO strain HST 2160.

6.8.3.1 Step 2 - GAP function

Edit and check sequences obtained by forward and reverse primers using the GAP function

GAP of: 2160t.seq check: 7731 from: 1 to: 458 to: 2160r.rev check: 7355 from: 1 to: 458

Gap Weight: 5.000	*Average Match: 1.000*
Length Weight: 0.300	*Average Mismatch: 0.000*
Quality: 457.0	*Length: 458*
Ratio: 0.998	*Gaps: 0*
Percent Similarity: 99.782	*Percent Identity: 99.127*

```
        .         .         .         .         .
  1 CATGCAAGTCGAGGGGAAACSGCATTAAGTGCTTGCACTTTTTGGACGTC 50
    ||||||||||||||||||||:||||||||||||||||||||||||||||
  1 CATGCAAGTCGAGGGGAAACGGCATTAAGTGCTTGCACTTTTTGGACGTC 50
        .         .         .         .         .
 51 GACCGGCGCACGGGTGAGTATCGCGTATCCAACCTGCCCATTACTTGGGG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 GACCGGCGCACGGGTGAGTATCGCGTATCCAACCTGCCCATTACTTGGGG 100
        .         .         .         .         .
101 ATAACCCGTTGAAAGACGGACTAATACCCGATGCAGTCCATTGAAGACAT 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 ATAACCCGTTGAAAGACGGACTAATACCCGATGCAGTCCATTGAAGACAT 150
        .         .         .         .         .
151 CTGAAGCGGACGAAAGATTTTATCGGTATTGGATGGGGATGCGTCCGATT 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 CTGAAGCGGACGAAAGATTTTATCGGTATTGGATGGGGATGCGTCCGATT 200
        .         .         .         .         .
201 AGCTTGTTGGCGGGGTAACGGCCCACCAAGGCATSSATCGGTAGGGGTTC 250
    ||||||||||||||||||||||||||||||||::||||||||||||||
201 AGCTTGTTGGCGGGGTAACGGCCCACCAAGGCATGCATCGGTAGGGGTTG 250
        .         .         .         .         .
251 TGAGAGGAAGGTCCCCCACATAGGAACTGAGACACGGTCCTAACTCCTAC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TGAGAGGAAGGTCCCCCACATAGGAACTGAGACACGGTCCTAACTCCTAC 300
        .         .         .         .         .
301 GGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGGTAGCCTGAACCAGC 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 GGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGGTAGCCTGAACCAGC 350
        .         .         .         .         .
351 CAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGC 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 CAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGC 400
        .         .         .         .         .
401 GGGGATAAAGTCACTCACGTGTGAGTGTTTGCAGTTACCGCATGAATAAG 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 GGGGATAAAGTCACTCACGTGTGAGTGTTTGCAGTTACCGCATGAATAAG 450

451 GACCGGCT 458
    ||||||||
451 GACCGGCT 458
```

6.8.3.2 Step 3 - FASTA search

Output generated by FASTA search to provide most likely relationship, demonstrated by PINLO HST 2160. The top row represents the rDNA sequence of PINLO HST 2160.

(Nucleotide) FASTA of: 2160t.seq from: 1 to: 458 January 11, 1998 14:04 (K. Robertson (10.1.98) PINLO 2160 TPU) TO: GenEMBL: Sequences:*
Mean score calculations exclude scores greater than 125
mean initn score: 33.4 (s.d. 10.03)
mean init1 score: 33.3 (s.d. 9.79)
1562 scores better than 597 saved, joining threshold: 50

The best scores are:		*init1*	*initn*	*opt..*
em_ba:pvorr16sa	*L16465 Prevotella corporis (ATCC 33547) ...*	*1087*	*1633*	*1634*
em_ba:pvorr16sb	*L16466 Prevotella denticola (ATCC 33185)...*	*982*	*1429*	*1424*
em_ba:pnrrna	*X73963 P. nigrescens gene for 16S rRNA.*	*1423*	*1423*	*1460*
em_ba:pvorr16sc	*L16467 Prevotella denticola (ATCC 35308)...*	*970*	*1409*	*1407*
em_ba:pvorr16so	*L16479 Prevotella nigrescens (ATCC 25261...*	*824*	*1394*	*1436*

ID PVORR16SA - *Prevotella corporis* (ATCC 33547) 16S ribosomal RNA.
SCORES Init1: 1087 Initn: 1633 Opt: 1634
93.4% identity in 458 bp overlap

```
                                        10        20        30
                                       CATGCAAGTCGAGGGGAAACSGCATTAAGT
                                       :::::::::::::::::::::::::::::
TCAGGATNAACGCTAGCTACAGGCTTAACACATGCAAGTCGAGGGGAAACGGCATTAAGT
         30        40        50        60        70        80

         40        50        60        70        80        90
GCTTGCACTTTTTGGACGTCGACCGGCGCACGGGTGAGTATCGCGTATCCAACCTGCCCA
:::::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::
GCTTGCACTTTTTGGACGTCGACCGGCGCACGGGTGAGTAACGCGTATCCAACCTGCCCC
         90        100       110       120       130       140

        100       110       120       130       140       150
TTACTTGGGGATAACCCGTTGAAAGACGGACTAATACCCGATGCAGTCCATTGAAGACAT
::::  ::: ::::::::::::::::::::::::: ::: ::: ::::: :::: : :::
TTACCAGGGAATAACCCGTTGAAAGACGGACTAATGCCCTATGGAGTCCTTTGACGGCAT
        150       160       170       180       190       200

        160       170       180       190       200       210
CTGAAGCGGACGAAAGATTTTATCGGTATTGGATGGGGATGCGTCCGATTAGCTTGTTGG
: ::    :::: ::::: :: :::::::: :::::::::::::: ::::::::::::::
CAGATTAGGACTAAAGA-TTCATCGGTATGGGATGGGGATGCGTCTGATTAGCTTGTTGG
       210       220       230       240       250       260

        220       230       240       250       260       270
CGGGGTAACGGCCCACCAAGGCATSSATCGGTAGGGGTTCTGAGAGGAAGGTCCCCCACA
:::::::::::::::::::::::::::::: :::: ::::::::::::::::::::::::::::::
CGGGGTAACGGCCCACCAAGGCATCGATCAGTAGGGGTTCTGAGAGGAAGGTCCCCCACA
        270       280       290       300       310       320
```

```
       280        290        300        310        320        330
TAGGAACTGAGACACGGTCCTAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAAT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TAGGAACTGAGACACGGTCCTAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAAT
       330        340        350        360        370        380

       340        350        360        370        380        390
GGGCGGTAGCCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAACT
|||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGGCGCTAGCCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAACT
       390        400        410        420        430        440

       400        410        420        430        440        450
GCTTTTATGCGGGGATAAAGTCACTCACGTGTGAGTGTTTGCAGTTACCGCATGAATAAG
:|||||||||||||||||||||||  ||||||||  ||:||||||||  ||||||||||||||
NCTTTTATGCGGGGATAAAGTCACCCACGTGTGGGTNTTTGCAGGTACCGCATGAATAAG
       450        460        470        480        490        500

GACCGGCT
||||||||
GACCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTCCGGGCGTTATCCGGAT
       510        520        530        540        550        560
```

6.8.3.3 Step 6 - Removal of uncertain bases from aligned sequences

LINEUP was used to remove uncertain base positions from the aligned sequences. These were primarily regions of insertions or deletions, resulting in an absence of informative base at that position. In some cases bases to either side of the insertion were removed to enable correct alignment of sequences. The relevant regions of the alignment are shown below. Areas in bold represent those that were removed prior to distance calculation. For key see end of section 6.8.3.3.

```
     101                                                        150
Pp1  CGTCGACCGG CGCACGGGTG AGTA.TCGCG TATCCAACCT GCCCTTTACT
Pp2  CGTCGACCGG CGCACGGGTG AGTATTCGCG TATCCAACCT GCCCTTTAAC
Pp3  CGTCGACCGG CGCACGGGTG AGTA.TCGCG TATCCAACCT GCCCTTTACT
Pn1  CGTTGACCGG CGCACGGGTG AGTA.TCGCG NATCCAACCT GCCCCNTACT
Pn2  CGTTGACCGG CGCACGGGTG AGTA.TCGCG NATCCAACCT GCCCCNNACT
Pi   CGTCGACCGG CGCACGGGTG AGTA.TCGCG NATCCAACCT TCCCTCCACT
Pd1  CGTCGACCGG CGCACGGGTG AGTA.ACGCG TATCCAACCT TCCCGTTACT
Pd2  CGTCGACCGG CGCACGGGTG AGTA.ACGCG TATCCAACCT TCCCGTTACT
156  CGTCGACCGG CGCACGGGTG AGTA.TCGCG TATCCAACCT GCCCATTACT
160  CGTCGACCGG CGCACGGGTG AGTA.TCGCG TATCCAACCT GCCCATTACT
391  CgTCKACCGG CGCACGGGTG AGTA.TCGCG TATCCAACCT GCCCATTACT
Pc   CGTCGACCGG CGCACGGGTG AGTA.ACGCG TATCCAACCT GCCCCTTACC

     151                                                        200
Pp1  TGGGGGATAA CCCCGTTGAA AGACGGCCTA ATAACCCGAT GTAATTCATT
Pp2  TTGGGGAT.A CCCCGTTGAA AGACGGCCTA AT.ACCCGAT GTAATTCATT
Pp3  TGGGG..ATA CCCCGTTGAA AGACGGCCTA ATAACCCGAT GTAATTCATT
Pn1  TGGGG..ATA CCCCGTTGAA AGACGGCCTN AT.ACCCGAT GTGTTTCATT
Pn2  TGGGG..ATA CCCCGTTGAA AGACGGCCTN AT.ACCCGAT GTGTTTCATT
Pi   CGGGG..ATA CCCCGTTGAA AGACGGCCTA AT.ACCCGAT GTTGTCCACA
Pd1  GCGGG..ATA ACCTGCCGAA AGGCNGACTA AT.ACCGCAT GTTCTTCGAT
Pd2  GCGGG..ATA ACCTGCCGAA AGGCAGACTA AT.ACCGCAT GTTCTTCGAT
156  TGGGG..ATA ACCCGTTGAA AGACGGACTA AT.ACCCGAT GCAGTCCATT
160  TGGGG..ATA ACCCGTTGAA AGACGGACTA AT.ACCCGAT GCAGTCCATT
391  TGGGG..ATA ACCCGTTGAA ARACGGACTA AT.ACCCGAT GCAGTCCATT
Pc   AGGGA..ATA ACCCGTTGAA AGACGGACTA AT.GCCCTAT GGAGTCCTTT

     201                                                        250
Pp1  GATGGCATCA GATATGAATA AAAGATTTA. TCGGTAAAGG ATGGGGATGC
Pp2  GATGGCATCA GATATGAATA AAAGATTTA. TCGGTAAAGG ATGGGGATGC
Pp3  GATGGCATCA GATATGAATA AAAGATTTAT TCGGTAAASG ATGGGGATGC
Pn1  GACGGCATCC GATATGAAAC AAAGGTTTT. CCGGTAAGGG ATGGGGATGC
Pn2  GACGGCATCC GATATGAAAC AAAGGTNTT. CCGGTAAGGG ATGGGGATGC
Pi   TATGGCATCT GACGTGGACC AAAGATTCA. TCGGTGGAGG ATGGGGATGC
Pd1  GACGGCATCA GATTCGAAGC AAAGA.TCCG TCGGTAACGG AGGGGGATGC
Pd2  GACGGCATCA GATTNGAAGC AAAGA.TCCR TCGGTAACGG AGGGGGATGC
156  GAAGACATCT GAAGCGGACG AAAGATTTTA TCGGTATTGG ATGGGGATGC
160  GAAGACATCT GAAGCGGACG AAAGATTTTA TCGGTATTGG ATGGGGATGC
391  GAAGACATCT GAAGCGGACR AAAGATTTTA TCGGTATTGG ATGGGGATGC
Pc   GACGGCATCA GATTAGGACT AAAGA.TTCA TCGGTATGGG ATGGGGATGC
```

```
     251                                                        300
Pp1  GTCTGATTAG CTTGTTGGTG .AGGTAAAGG CTCACCAAGG CNACGATCAG
Pp2  GTCTGATTAG CTTGTTGGTG .AGGTAAAGG CTCACCAAGG CGACGATCAG
Pp3  GTCTGATTAG CTTGTTGGTG .AGGTAAAGG CTCACCAAGG CNACGATCAG
Pn1  GTCTGATTAG CTNGTTGGCG .GGGCAACGG CCCACCAAGG CGACGATCAG
Pn2  GTCTGATTAG CTTGTTGGCG .GGGCAACGG CCCACCAAGG CGACGATCAG
Pi   GTCTGATTAG CTTGTTGGTG CGGGTAACGG CCCACCAAGG CTNCGATCAG
Pd1  GTCTGATTAG CTAGTTGGCG .GGGCGACGG CCCACCAAGG CGACGATCAG
Pd2  GTCTGATTAG CTAGTTGGCG .GGGCGACGG CCCACCAAGG CGACGATCAG
156  GTCCGATTAG CTTGTTGGCG .GGGTAACGG CCCACCAAGG CATCGATCGG
160  GTCCGATTAG CTTGTTGGCG .GGGTAACGG CCCACCAAGG CATSSATCGG
391  GTCCGATTAG CTTGTTGGCG .GGGTAACGG CCCACCAAGG CATCGATCGG
Pc   GTCTGATTAG CTTGTTGGCG .GGGTAACGG CCCACCAAGG CATCGATCAG

     451                                                        500
Pp1  CTTTTATACG AGAATAATTT GATGCACGTG TGCGTTATTG CATGTATCGT
Pp2  CTTTTATACG AGAATAATTT GATGCACGTG TGCGTTATTG CATGTATCGT
Pp3  CTTTTATACG AGAATAATTT GATGCACGTG TGCGTTATTG CATGTATCGT
Pn1  CNTTTATGTG GGAATAAA.. T.TGCACGTG TGCGCNNTTG CATGTACCTC
Pn2  CNTTTATGTG GGAATAAA.. G.TGCACGTG TGCGCNNTTG CATGTACCTC
Pi   CTTTTGTTGG GGAGTAAAGC G.GGCACGTG TGCCNNNTTG CATTTACCCT
Pd1  CTTTTATGCG GGGATAAAGT GAGGGACGNN NNNNNNTTTG CAGGTACCGC
Pd2  CTTTTATGCG GGGATAAAGT GAGGNACGNN NCCNNNTTTG CAGGTACCGC
156  CTTTTATGCG GGGATAAAGT CACTCACGTG TGAGTGTTTG CAGTTACCGC
160  CTTTTATGCG GGGATAAAGT CACTCACGTG TGAGTGTTTG CAGTTACCGC
391  CTTTTATGCG GGGATAAAGT CACTCACGTG TGAGTGTTTG CAGTTACCGC
Pc   CTTTTATGCG GGGATAAAGT CACCCACGTG TGGGTNTTTG CAGGTACCGC
```

KEY:

*Pp*1, *P. pallens* strain 1037; *Pp*2, *P. pallens* strain 9423; *Pp*3, *P. pallens* strain 8792; *Pn*1, *P. nigrescens* ATCC 33563; *Pn*2, *P. nigrescens* ATCC 25261; *Pi*, *P. intermedia* ATCC 25611, *Pd*1, *P. denticola* ATCC 33185; *Pd*2, *P. denticola* ATCC 35308; 156, PINLO HST 1156; 160, PINLO HST 2160; 391, PINLO A391; *Pc*, *P. corporis* ATCC 33547.

6.8.3.4 Step 7 - Calculation of distances between nucleic acid sequences

Using DISTANCES command to measure dissimilarities between nucleic acid sequences. A distance matrix is produced. For key see section 6.8.3.3.

DISTANCES between nucleic acid sequences

Calculated over: 1 to 441, considering all base positions

Correction method: Jukes-Cantor

Distances are: estimated number of substitutions per 100 bases

	Pp1	*Pp2*	*Pp3*	*Pn1*	*Pn2*	*Pi*	*Pd1*	*Pd2*	156	160	391	*Pc*
Pp1	0.00	0.91	0.91	8.39	8.64	11.50	13.10	13.37	10.19	10.19	11.50	10.19
Pp2		0.00	1.84	8.90	9.15	12.29	13.37	13.64	10.97	10.97	12.29	10.45
Pp3			0.00	8.90	9.15	11.76	13.91	14.19	10.97	10.97	12.29	11.23
Pn1				0.00	0.68	10.19	12.03	12.29	11.23	11.23	12.56	10.45
Pn2					0.00	10.19	12.29	12.56	11.23	11.23	12.56	10.45
Pi						0.00	16.41	16.69	12.56	12.56	13.91	13.91
Pd1							0.00	1.60	12.03	12.03	13.37	10.71
Pd2								0.00	12.83	12.83	14.19	11.23
156									0.00	0.00	1.14	5.41
160										0.00	1.14	5.41
391											0.00	6.63
Pc												0.00

This information is represented in dendrogram V.

Description of Jukes-Cantor distance correction:

Jukes-Cantor distance assumes that a nucleic acid substitution can occur at any site with equal probability and that when it does occur, the probability of change to any of the other three nucleotides is equal. These assumptions break down with increasing sequence divergence, therefore this correction is suitable for closely related sequences (Genhelp).

6.8.3.5 Step 8 - Construction of a dendrogram representing the distances between nucleic acid sequences

The distance matrix is entered into a syntax file in SPSS for windows version 6.1 in the following way. This dendrogram is shown in figure 3.12.

```
matrix data variables=rowtype_ pp pp2 pp3 pn pn2 pi pd pd2 pin1 pin2 pin3 pc.
begin data
prox  .00
prox  .91    .00
prox  0.91   1.84   .00
prox  8.39   8.90   8.90   .00
prox  8.64   9.15   9.15   0.68  .00
prox  11.50  12.29  11.76  10.19 10.19 .00
prox  13.10  13.37  13.91  12.03 12.29 16.41 .00
prox  13.37  13.64  14.19  12.29 12.56 16.69 1.60  .00
prox  10.19  10.97  10.97  11.23 11.23 12.56 12.03 12.83 .00
prox  10.19  10.97  10.97  11.23 11.23 12.56 12.03 12.83 .00  .00
prox  11.50  12.29  12.29  12.56 12.56 13.91 13.37 14.19 1.14 1.14 .00
prox  10.19  10.45  11.23  10.45 10.45 13.91 10.71 11.23 5.41 5.41 6.63 .00
end data.
execute.
value labels rowtype_ 'PROX' 'dissimilarity'.

CLUSTER
 /METHOD BAVERAGE
 /MEASURE= EUCLID
 /PRINT SCHEDULE
 /PLOT =DENDROGRAM
 /matrix=in(*).
```

6.9 ALIGNMENT OF SEQUENCES USED FOR DISTANCE CALCULATIONS

6.9.1 *P. INTERMEDIA*, *P. NIGRESCENS* AND CLINICAL ISOLATES A-D

Only informative bases are included in this alignment, insertions and deletions have been removed and where possible, ambiguous bases have been assigned. Bases in bold represent differences between other bases in the same position.

This information was used to calculate the distance matrix (see section 6.10.1) from which dendrogram IV was derived.

KEY:

*Pn*1a *P. nigrescens* ATCC 33563 (EMBL); *Pn*2a *P. nigrescens* ATCC 25261 (EMBL); *Pn*5, *P. nigrescens* MH5; *Pn*94, *P. nigrescens* LM94; A, PLS isolate **1***2B P. nigrescens*; D, PLS isolate **3***2A P. nigrescens*; *Pn*2b *P. nigrescens* MH2; *Pn*4, *P. nigrescens* MH4; *Pn*1b *P. nigrescens* MH1; *Pn*3, *P. nigrescens* NCTC 9336 (EMBL); *Pi*6, *P. intermedia* MH6; *Pi*12, *P. intermedia* MH12; B, PLS isolate **2***3A P. intermedia*; *Pi*3, *P. intermedia* MH3; *Pi*2, *P. intermedia* ATCC 25611 (EMBL); C, PLS isolate **5***1D P. intermedia*; *Pi*15, *P. intermedia* MH15; *Pi*1, *P. intermedia* ATCC 25611 (EMBL).

```
     1                                                  50
Pn1a TTATGTGCTT GCACATTCTG GACGTTGACC GGCGCACGGG TGAGTATCGC
Pn2a TTATGTGCTT GCACATTCTG GACGTTGACC GGCGCACGGG TGAGTATCGC
Pn5  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pn94 TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
12B  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
32A  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pn2b TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pn4  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pn1b TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pn3  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi6  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi12 TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
23A  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi3  TTATGTGCTT GCACATTTTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi2  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
51D  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi15 TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
Pi1  TTATGTGCTT GCACATTCTG GACGTCGACC GGCGCACGGG TGAGTATCGC
```

```
     51          a    bc    d                              100
Pn1a GTATCCAACC TGCCCCNTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn2a GTATCCAACC TGCCCCNNAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn5  GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn94 GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
12B  GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
32A  GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn2b GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn4  GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn1b GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pn3  GTATCCAACC TGCCCCTTAC TTGGGGATAC CCCGTTGAAA GACGGCCTAA
Pi6  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
Pi12 GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
23A  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
Pi3  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
Pi2  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
51D  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA
Pi15 GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GGCGGCCTAA
Pi1  GTATCCAACC TTCCCTCCAC TCGGGGATAC CCCGTTGAAA GACGGCCTAA

     101         ef g  hij  k      l   mn  o p     q    rst150
Pn1a TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
Pn2a TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
Pn5  TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
Pn94 TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
12B  TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
32A  TACCCGATGT GTTTCATTGA CGGCATCCGA TRTGAAACAA AGGCGGTAAG
Pn2b TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
Pn4  TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAR
Pn1b TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACAA AGGCGGTAAG
Pn3  TACCCGATGT GTTTCATTGA CGGCATCCGA TATGAAACCA AAGCGGTAAG
Pi6  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
Pi12 TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
23A  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
Pi3  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
Pi2  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
51D  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
Pi15 TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
Pi1  TACCCGATGT TGTCCACATA TGGCATCTGA CGTGGACCAA AGACGGTGGA
```

```
     151                                        u     v              200
Pn1a GGATGGGGAT GCGTCTGATT AGCTNGTTGG CGGGGCAACG GCCCACCAAG
Pn2a GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
Pn5  GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
Pn94 GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
12B  GGATGGGGAT GCGTCTGATT AGCTAGTTGG CGGGGCAACG GCCCACCAAG
32A  GGATGGGGAT GCGTCTGATT AGCTAGTTGG CGGGGCAACG GCCCACCAAG
Pn2b GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
Pn4  GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
Pn1b GGATGGGGAT GCGTCTGATT AGCTTGTTGG CGGGGCAACG GCCCACCAAG
Pn3  GGATGGGGAT GCGTCTGATT AGCTAGTTGG CGGGGCAACG GCCCACCAAG
Pi6  GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
Pi12 GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
23A  GGATGGGGAT GCGTCTGATT AGCTKGTTGG TGGGGTAACG GCCCACCAAG
Pi3  GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
Pi2  GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
51D  GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
Pi15 GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG
Pi1  GGATGGGGAT GCGTCTGATT AGCTTGTTGG TGGGGTAACG GCCCACCAAG

     201                                                   250
Pn1a GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn2a GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn5  GCRACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn94 GCRACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
12B  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
32A  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn2b GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn4  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn1b GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pn3  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pi6  GCAACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pi12 GCTACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
23A  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTcCCCCACA TTGGAACTGA
Pi3  GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pi2  GCTACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
51D  GCTACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pi15 GCGACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
Pi1  GCTACGATCA GTAGGGGTTC TGAGAGGAAG GTCCCCCACA TTGGAACTGA
```

```
     251                                                        300
Pn1a GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn2a GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn5  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn94 GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
12B  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
32A  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn2b GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn4  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn1b GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pn3  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi6  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi12 GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
23A  GACACGGTcc AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi3  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi2  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
51D  GAcACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi15 GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT
Pi1  GACACGGTCC AAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT

     301  w                                                     350
Pn1a GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn2a GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn5  GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn94 GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
12B  GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
32A  GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn2b GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn4  GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn1b GGACGCAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pn3  GGACGYAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi6  GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi12 GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
23A  GGACGGAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi3  GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi2  GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
51D  GGACGKAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi15 GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
Pi1  GGACGTAAGT CTGAACCAGC CAAGTAGCGT GCAGATGACG GCCCTATGGG
```

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     351                 xy     z             388
Pn1a TTGTAAACTG CTTTTATGTG GGAATAAATT GCACGTGT
Pn2a TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
Pn5  TTGTAAACTG CTTTTATGTG GGAATAAATT GCACGTGT
Pn94 TTGTAAACTG CTTTTATKTG GGAATAAAKT GCACGTGT
12B  TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
32A  TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
Pn2b TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
Pn4  TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
Pn1b TTGTAAACTG CTTTTATGTG GGAATAAATT GCACGTGT
Pn3  TTGTAAACTG CTTTTATGTG GGAATAAAGT GCACGTGT
Pi6  TTGTAAACTG CTTTTGTTGG GGAGTAAAGT GCACGCGT
Pi12 TTGTAAACTG CTTTTGTTGG GGAGTAAAGT GCACGCGT
23A  TTGTAAACTG CTTTTGTTGG GGAGTAAAGT GCACGCGT
Pi3  TTGTAAACTG CTTTTGTTGG GGAGTAAAGT GCACGTGT
Pi2  TTGTAAACTG CTTTTGTTGG GGAGTAAAGC GCACGTGT
51D  TTGTAAACTG CTTTTGTTGG GGAKTAAAGT GCACGTGT
Pi15 TTGTAAACTG CTTTTGTTGG GGAGTAAAGT GCACGCGT
Pi1  TTGTAAACTG CTTTTGTTGG GGAGTAAAGC GCACGTGT
```

6.9.2 PINLOs

For key see section 6.8.3.3.

```
     1                                                            50
Pp1  CATGCAAGTC GAGGGGAAAC GGCATTATGT GCTTGCACAT TTTGGACGTC
Pp2  CATGCAAGTC GAGGGGAAAC GGCATTATGT GCTTGCACAT TTTGGACGTC
Pp3  CATGCAAGTC GAGGGGAAAC GGCATTATGT GCTTGCACAT TTTGGACGTC
Pn1  CATGCAAGTC GTGGGGAAAC GGCATTATGT GCTTGCACAT TCTGGACGTT
Pn2  CATGCAAGTC GTGGGGAAAC GGCATTANGT GCTTGCACAT TCTGGACGTT
Pi   CATGCAAGTC GAGGGGAAAC GGCATTATGT GCTTGCACAT TCTGGACGTC
Pd1  CATGCAAGTC GAGGGGAAAC GGCATTGAGT GCTTGCACTC AATGGACGTC
Pd2  CATGCAAGTC GAGGGGAAAC GGCATTGAGT GCTTGCACTN AATGGACGTC
156  CATGCAAGTC GAGGGGAAAC GGCATTAAGT GCTTGCACTT TTTGGACGTC
160  CATGCAAGTC GAGGGGAAAC GGCATTAAGT GCTTGCACTT TTTGGACGTC
391  CATGCAAGTC GAGGGGAAAC GGCATTAAGT GCTTGCACTT TTTGGACGTC
Pc   CATGCAAGTC GAGGGGAAAC GGCATTAAGT GCTTGCACTT TTTGGACGTC

     51                                                           100
Pp1  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCT TTACTTGGGG
Pp2  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCT TTAACTTGGG
Pp3  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCT TTACTTGGGG
Pn1  GACCGGCGCA CGGGTGAGTA TCGCGNATCC AACCTGCCCC NTACTTGGGG
Pn2  GACCGGCGCA CGGGTGAGTA TCGCGNATCC AACCTGCCCC NNACTTGGGG
Pi   GACCGGCGCA CGGGTGAGTA TCGCGNATCC AACCTTCCCT CCACTCGGGG
Pd1  GACCGGCGCA CGGGTGAGTA ACGCGTATCC AACCTTCCCG TTACTGCGGG
Pd2  GACCGGCGCA CGGGTGAGTA ACGCGTATCC AACCTTCCCG TTACTGCGGG
156  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCA TTACTTGGGG
160  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCA TTACTTGGGG
391  GACCGGCGCA CGGGTGAGTA TCGCGTATCC AACCTGCCCA TTACTTGGGG
Pc   GACCGGCGCA CGGGTGAGTA ACGCGTATCC AACCTGCCCC TTACCAGGGA

     101                                                          150
Pp1  CCCGTTGAAA GACGGCCTAA TACCCGATGT AATTCATTGA TGGCATCAGA
Pp2  CCCGTTGAAA GACGGCCTAA TACCCGATGT AATTCATTGA TGGCATCAGA
Pp3  CCCGTTGAAA GACGGCCTAA TACCCGATGT AATTCATTGA TGGCATCAGA
Pn1  CCCGTTGAAA GACGGCCTAA TACCCGATGT GTTTCATTGA CGGCATCCGA
Pn2  CCCGTTGAAA GACGGCCTAA TACCCGATGT GTTTCATTGA CGGCATCCGA
Pi   CCCGTTGAAA GACGGCCTAA TACCCGATGT TGTCCACATA TGGCATCTGA
Pd1  CCTGCCGAAA GGCGGACTAA TACCGCATGT TCTTCGATGA CGGCATCAGA
Pd2  CCTGCCGAAA GGCAGACTAA TACCGCATGT TCTTCGATGA CGGCATCAGA
156  CCCGTTGAAA GACGGACTAA TACCCGATGC AGTCCATTGA AGACATCTGA
160  CCCGTTGAAA GACGGACTAA TACCCGATGC AGTCCATTGA AGACATCTGA
391  CCCGTTGAAA GACGGACTAA TACCCGATGC AGTCCATTGA AGACATCTGA
Pc   CCCGTTGAAA GACGGACTAA TGCCCTATGG AGTCCTTTGA CGGCATCAGA
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     151                                                        200
Pp1  TATGAATAAA AGACGGTAAA GGATGGGGAT GCGTCTGATT AGCTTGTTGG
Pp2  TATGAATAAA AGACGGTAAA GGATGGGGAT GCGTCTGATT AGCTTGTTGG
Pp3  TATGAATAAA AGACGGTAAA SGATGGGGAT GCGTCTGATT AGCTTGTTGG
Pn1  TATGAAACAA AGGCGGTAAG GGATGGGGAT GCGTCTGATT AGCTNGTTGG
Pn2  TATGAAACAA AGGCGGTAAG GGATGGGGAT GCGTCTGATT AGCTTGTTGG
Pi   CGTGGACCAA AGACGGTGGA GGATGGGGAT GCGTCTGATT AGCTTGTTGG
Pd1  TTCGAAGCAA AGACGGTAAC GGAGGGGGAT GCGTCTGATT AGCTAGTTGG
Pd2  TTNGAAGCAA AGACGGTAAC GGAGGGGGAT GCGTCTGATT AGCTAGTTGG
156  AGCGGACGAA AGACGGTATT GGATGGGGAT GCGTCCGATT AGCTTGTTGG
160  AGCGGACGAA AGACGGTATT GGATGGGGAT GCGTCCGATT AGCTTGTTGG
391  AGCGGACGAA AGACGGTATT GGATGGGGAT GCGTCCGATT AGCTTGTTGG
Pc   TTAGGACTAA AGACGGTATG GGATGGGGAT GCGTCTGATT AGCTTGTTGG

     201                                                        250
Pp1  TGAGGTAAAG GCTCACCAAG GCNACGATCA GTAGGGGTTC TGAGAGGAAG
Pp2  TGAGGTAAAG GCTCACCAAG GCGACGATCA GTAGGGGTTC TGAGAGGAAG
Pp3  TGAGGTAAAG GCTCACCAAG GCNACGATCA GTAGGGGTTC TGAGAGGAAG
Pn1  CGGGGCAACG GCCCACCAAG GCGACGATCA GTAGGGGTTC TGAGAGGAAG
Pn2  CGGGGCAACG GCCCACCAAG GCGACGATCA GTAGGGGTTC TGAGAGGAAG
Pi   TGGGGTAACG GCCCACCAAG GCTNCGATCA GTAGGGGTTC TGAGAGGAAG
Pd1  CGGGGCGACG GCCCACCAAG GCGACGATCA GTAGGGGTTC TGAGAGGAAG
Pd2  CGGGGCGACG GCCCACCAAG GCGACGATCA GTAGGGGTTC TGAGAGGAAG
156  CGGGGTAACG GCCCACCAAG GCATCGATCG GTAGGGGTTC TGAGAGGAAG
160  CGGGGTAACG GCCCACCAAG GCATCGATCG GTAGGGGTTC TGAGAGGAAG
391  CGGGGTAACG GCCCACCAAG GCATCGATCG GTAGGGGTTC TGAGAGGAAG
Pc   CGGGGTAACG GCCCACCAAG GCATCGATCA GTAGGGGTTC TGAGAGGAAG

     251                                                        300
Pp1  GTCCCCCACA TTGGAACTGA GACACGGTCC AAACTCCTAC GGGAGGCAGC
Pp2  GTCCCCCACA TTGGAACTGA GACACGGTCC AAACTCCTAC GGGAGGCAGC
Pp3  GTCCCCCACA TTGGAACTGA GACACGGTCC AAACTCCTAC GGGAGGCAGC
Pn1  GTCCCCCACA TTGGAACTGA GACACGGTCC NAACTCCTAC GGGAGGCAGC
Pn2  GTCCCCCACA TTGGAACTGA GACACGGTCC NAACTCCTAC GGGAGGCAGC
Pi   GTCCCCCACA TTGGAACTGA GACACGGTCC NAACTCCTAC GGGAGGCAGC
Pd1  GTCCCCCACA TTGGAACTGA GACACGGTCC AAACTCCTAC GGGAGGCAGC
Pd2  GTCCCCCACA TTGGAACTGA GACACGGTCC AAACTCCTAC GGGAGGCAGC
156  GTCCCCCACA TAGGAACTGA GACACGGTCC TAACTCCTAC GGGAGGCAGC
160  GTCCCCCACA TAGGAACTGA GACACGGTCC TAACTCCTAC GGGAGGCAGC
391  GTCCCCCACA TAGGAACTGA GACACGGTCC TAACTCCTAC GGGAGGCAGC
Pc   GTCCCCCACA TAGGAACTGA GACACGGTCC TAACTCCTAC GGGAGGCAGC

     301                                                        350
Pp1  AGTGAGGAAT ATTGGTCAAT GGGCGCAAGC CTGAACCAGC CAAGTAGCGT
Pp2  AGTGAGGAAT ATTGGTCAAT GGGCGCAAGC CTGAACCAGC CAAGTAGCGT
Pp3  AGTGAGGAAT ATTGGTCAAT GGNCGNAAGN CTGAACCAGC CAAGTAGCGT
Pn1  AGTGAGGAAT ATTGGTCAAT GGACGCAAGT CTGAACCAGC CAAGTAGCGT
Pn2  AGTGAGGAAT ATTGGTCAAT GGACGCAAGT CTGAACCAGC CAAGTAGCGT
Pi   AGTGAGGAAT ATTGGTCAAT GGACGTAAGT CTGAACCAGC CAAGTAGCGT
Pd1  AGTGAGGAAT ATTGGTCAAT GGGCGGAAGC CTGAACCAGC CAAGTAGCGT
Pd2  AGTGAGGAAT ATTGGTCAAT GGGCGGAAGC CTGAACCAGC CAAGTAGCGT
156  AGTGAGGAAT ATTGGTCAAT GGGCGGTAGC CTGAACCAGC CAAGTAGCGT
160  AGTGAGGAAT ATTGGTCAAT GGGCGGTAGC CTGAACCAGC CAAGTAGCGT
391  AGTGAGGAAT ATTGGTCAAT GGGCGGTAGC CTGAACCAGC CAAGTAGCGT
Pc   AGTGAGGAAT ATTGGTCAAT GGGCGCTAGC CTGAACCAGC CAAGTAGCGT
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     351                                                        400
Pp1  GCAGGAAGAC GGCCCTATGG GTTGTAAACT GCTTTTATAC GAGAATAATC
Pp2  GCAGGAAGAC GGCCCTATGG GTTGTAAACT GCTTTTATAC GAGAATAATC
Pp3  GCAGGAAGAC GGCCCTATGG GTTGTAAACT GCTTTTATAC GAGAATAATC
Pn1  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGT GGGAATAAAC
Pn2  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGT GGGAATAAAC
Pi   GCAGATTGAC GGCCCTATGG GTTGTAAACT GCTTTTGTTG GGGAGTAAAC
Pd1  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGC GGGGATAAAG
Pd2  GCAGGATGAC GGCCCTACGG GTTGTAAACT GCTTTTATGC GGGGATAAAN
156  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGC GGGGATAAAC
160  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGC GGGGATAAAC
391  GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGC GGGGATAAAC
Pc   GCAGGATGAC GGCCCTATGG GTTGTAAACT GCTTTTATGC GGGGATAAAC

     401                                                441
Pp1  ACGTGTGCGT TATTGCATGT ATCGTATGAA TAAGGACCGG C
Pp2  ACGTGTGCGT TATTGCATGT ATCGTATGAA TAAGGACCGG C
Pp3  ACGTGTGCGT TATTGCATGT ATCGTATGAA TAAGGACCGG C
Pn1  ACGTGTGCGC NNTTGCATGT ACCTCATGAA TAAGGACCGG C
Pn2  ACGTGTGCGC NNTTGCATGT ACCTCATGAA TAAGGACCGG C
Pi   ACGTGTGCCN NNTTGCATTT ACCCTTCGAA TAAGGACCGG C
Pd1  ACGTGTNNNN NTTTGCAGGT ACCGCATGAA TAAGGACCGG C
Pd2  ACGTGTCCNN NTTTGCAGGT ACCGCATGAA TAAGGACCGG C
156  ACGTGTGAGT GTTTGCAGTT ACCGCATGAA TAAGGACCGG C
160  ACGTGTGAGT GTTTGCAGTT ACCGCATGAA TAAGGACCGG C
391  ACGTGTGAGT GTTTGCAGTT ACCGCATGAA TAAGACCGGC T
Pc   ACGTGTGGGT NTTTGCAGGT ACCGCATGAA TAAGGACCGG C
```

6.10 DISTANCE MATRICES

6.10.1 *P. INTERMEDIA* AND *P. NIGRESCENS*

The information tabulated below is represented in dendrogram IV (figure 3.13). For key see section 6.9.1.

DISTANCES between nucleic acid sequences

Calculated over: 1 to 388, considering all base positions

Correction method: Jukes-Cantor

Distances are: estimated number of substitutions per 100 bases

	Pn 1a	*Pn* 2a	*Pn* 5	*Pn* 94	1 2B	3 2A	*Pn* 2b	*Pn* 4	*Pn* 1B	*Pn* 3	*Pi* 6	*Pi* 12	2 3A	*Pi* 3	*Pi* 2	5 1D	*Pi* 15	*Pi* 1
*Pn*1a	0.00	0.78	1.04	1.56	1.04	1.30	1.04	1.30	0.78	1.83	9.03	9.03	9.03	8.74	9.03	8.74	9.03	9.03
*Pn*2a		0.00	1.30	1.56	1.04	1.30	0.78	1.04	1.04	1.83	8.45	8.45	8.74	8.16	8.45	8.16	8.45	8.45
*Pn*5			0.00	0.52	0.78	1.04	0.52	0.78	0.26	1.56	8.45	8.45	9.03	8.45	8.45	8.16	8.74	8.45
*Pn*94				0.00	1.04	1.30	0.78	1.04	0.78	1.83	8.45	8.45	9.03	8.45	8.45	8.16	8.74	8.45
12B					0.00	0.26	0.26	0.52	0.52	0.78	8.45	8.45	8.45	8.16	8.45	8.16	8.45	8.45
32A						0.00	0.52	0.78	0.78	1.04	8.45	8.45	8.45	8.16	8.45	8.16	8.45	8.45
*Pn*2b							0.00	0.26	0.26	1.04	8.16	8.16	8.45	7.87	8.16	7.87	8.16	8.16
*Pn*4								0.00	0.52	1.30	8.16	8.16	8.45	7.87	8.16	7.87	8.16	8.16
*Pn*1B									0.00	1.30	8.45	8.45	8.74	8.16	8.45	8.16	8.45	8.45
*Pn*3										0.00	9.03	9.03	9.03	8.74	9.03	8.74	9.03	9.03
*Pi*6											0.00	0.26	1.04	0.78	0.78	1.04	0.52	0.78
*Pi*12												0.00	1.04	0.78	0.52	0.78	0.52	0.52
23A													0.00	1.04	1.56	1.56	1.04	1.56
*Pi*3														0.00	0.78	1.04	0.78	0.78
*Pi*2															0.00	0.78	1.04	0.00
51D																0.00	1.30	0.78
*Pi*15																	0.00	1.04
*Pi*1																		0.00

6.10.2 PINLOs

The distance matrix used for dendrogram V (figure 3.14) is included in section 6.8.3.5 as an example of manipulation of sequence data.

6.10.3 PLS ISOLATES

6.10.3.1 *Actinomyces* sp.

KEY:

Ai; *A. israelii*, **1** *1B; A. israelli*, **3** *1A; Actinomyces sp., Am*; *A. meyeri*, *Ao; A. odontolyticus, An*; *A. naeslundii, A v*; *A. viscosus, Ah*; *A. howelli.*

	Ai	**1** *1B*	**3** *1A*	*Am*	*Ao*	*An*	*Av*	*An*	*Ah*	*Av*	*Ai.*
Ai	0.00	**2.00**	**14.38**	22.39	22.99	15.85	17.31	15.71	14.61	29.39	14.08
1 *1B*		0.00	**16.10**	23.57	24.71	17.30	18.23	17.32	15.66	31.86	15.71
3 *1A*			0.00	23.46	23.19	19.60	18.85	18.01	17.35	29.78	24.99
Am.				0.00	4.12	16.93	17.48	18.11	20.02	29.69	24.49
Ao.					0.00	16.78	16.52	18.45	18.10	29.28	24.87
An						0.00	3.35	0.77	11.11	18.15	14.68
Av							0.00	2.99	10.73	15.17	16.34
An								0.00	8.74	18.56	16.34
Ah.									0.00	21.42	14.77
Av										0.00	31.98
Ai.											0.00

6.10.3.2 *Bacillus* sp.

KEY:

Bc; *B. cereus*, **15** *1A*; *B. cereus, Ba*; *B. anthracis, Bs*; *B. subtilis, Bd*; *B. denitrificans*

	Bc	**15** *1A*	*Bc*	*Ba*	*Bc*	*Bs*	*Bd*
Bc	0.00	**0.00**	0.42	0.83	0.63	8.37	12.87
15 *1A*		0.00	0.43	0.86	0.65	8.43	13.10
Bc			0.00	1.39	0.99	8.44	12.97
Ba				0.00	1.19	8.89	13.70
Bc					0.00	8.67	13.21
Bs						0.00	13.77
Bd							0.00

6.10.3.3 *Kingella* and *Suttonella* sp.

KEY:

Kd; *K. denitrificans*, **16** *1A*; *S. indologenes*, *Ko*; *K. oralis*, *Kk*; *K. kingae*, *Si*; *S. indologenes*; **3** *1E*; *S. indologenes*.

	Kd	**16** *1A*	*Ko*	*Kk*	*Si*	**3** *1E*
Kd	0.00	**1.85**	13.70	9.90	21.56	21.65
16 *1A*		0.00	14.85	11.85	22.14	22.21
Ko			0.00	8.99	25.81	27.80
Kk				0.00	26.32	28.77
Si					0.00	**14.48**
3 *1E*						0.00

6.10.3.4 *Leuconostoc* sp.

KEY:

Lcr; *L. cremoris*, *Lm*; *L. mesenteroides*, **9** *2E*; *Leuconostoc* sp., *Lp*; *L. paramesenteroides*, *Ll*; *L. lactis*, *La*; *L. amelobiosum*, *Lc*; *L. carnosum*, *Lf*; *L. fallax*

	Lcr	*Lm*	**9** *2E*	*Lm*	*Lp*	*Ll*	*La*	*Lc*	*Lf*
Lcr	0.00	0.00	**0.66**	2.77	2.77	5.49	17.68	9.79	14.54
Lm		0.00	**0.66**	2.77	2.77	5.49	17.68	9.79	14.54
9 *2E*			0.00	3.04	3.04	6.14	19.04	9.82	15.73
Lm.				0.00	3.03	6.46	10.62	8.39	11.19
Lp.					0.00	5.92	12.06	8.67	12.06
Ll.						0.00	17.43	10.65	17.97
La							0.00	11.22	21.56
Lc								0.00	15.37
Lf									0.00

6.10.3.5 *Neisseria* species

KEY:

9 *2A*; *N. elongata*, **14** *2A*; *N. elongata*, *Nf*; *N. flavescens*, *Np; N. polysaccharea*; *Nd*; *N. denitrificans*, *Ne*; *N. elongata*, ***3*** *2A*; *N. flavescens*, *Nl*; *N. lactamica.*

	9 *2A*	**14** *2A*	*Nf*	*Np*	*Nd*	*Nd*	*Ne*	***3*** *2A*	*Nl*
9 *2A*	0.00	0.00	**2.00**	4.05	9.06	9.31	8.81	8.81	9.80
14 *2A*		0.00	**1.99**	4.04	9.04	9.29	8.79	8.79	9.78
Nf			0.00	4.72	9.28	9.51	9.10	9.76	11.44
Np				0.00	8.85	9.08	9.77	10.49	10.19
Nd					0.00	0.40	6.88	6.90	10.68
Nd						0.00	7.10	7.14	10.68
Ne							0.00	**0.64**	7.99
3 *2A*								0.00	7.99
Nl									0.00

6.10.3.6 *Peptostreptococcus* sp.

KEY:

Pstr; *Peptostreptococcus* sp., *Pstra*; *Pstr. anaerobius*, **11** *1A*; *Peptostreptococcus* sp., *Pstm*; *Pstr. micros*

	Pstr	*Pstra*	*Pstra*	*Pstr*	**11** *1A*	*Pstr*	*Pstr*	*Pstrm*
Pstr	0.00	1.49	3.49	16.52	12.54	24.28	27.48	26.87
Pstra		0.00	2.59	16.71	13.25	24.16	27.66	26.73
Pstra			0.00	16.95	14.07	24.22	28.42	26.73
Pstr				0.00	17.01	32.07	30.06	37.88
11 *1A*					0.00	32.41	32.20	36.94
Pstr						0.00	15.89	22.22
Pstr.							0.00	22.64
Pstrm								0.00

6.11 EMBL INFORMATION FOR SPECIES USED DURING PCR PRIMER DESIGN

The 16S rRNA gene sequences of the species listed within table 6.3 were aligned and used to check the specificity of potential *P. intermedia* and *P. nigrescens* PCR primers (see section 2.7.2).

Table 6.3 EMBL identification and accession number of species used for sequence alignment of potential *P. intermedia* and *P. nigrescens* PCR primers

	ID	Accession No
A. actinomycetemcomitans	AARrNAM	M75036
B. forsythus strain 338	BFRR16SAV	L16495
F. nucleatum ATCC 25586	FNNSSRRNA	X55401
P. asaccharolytica ATCC 25260	PARR16SA	L16490
P. corporis ATCC 33547	PVORR16SA	L16465
P. dentalis DSM 3688	PDRRNA16S	X81879
P. denticola ATCC 33185	PVORR16SB	L16466
P. denticola ATCC 35308	PVORR16SC	L16467
P. endodontalis ATCC 35406	PERR16SB	L16491
P. gingivalis ATCC 33277	POYRR16SC	L16492
P. intermedia ATCC 25611	PVORR16SD	L16468
P. loescheii ATCC 15930	PVORR16SQ	L16481
P. melaninogenica ATCC 43982	PVORR16SF	L16470
P. nigrescens ATCC 25261	PVORR16SO	L16479
P. nigrescens ATCC 33563	PVORR16SG	L16471
P. oralis ATCC 33269	PVORR16SP	L16480
P. pallens strain 10371	PP16S1037	Y13105
P. pallens strain 8792	PP16S8792	Y13107
P. pallens strain 9423	PP16S9423	713106

6.12 ANNEALING POSITION OF *P. INTERMEDIA* AND *P. NIGRESCENS* PRIMERS IN THE 16S RRNA GENE IN RELATION TO RE-TPU1

The following sequence alignment shows the annealing positions of the PCR primers. The sequence of RE-TPU1, the forward primer was as shown below, primers P-int, P-nig, 1Bi-1 and 2Bi-1 were reversed.

```
           1                                                   50
Pi 25611   TACAATGGAG AGTTTGATCC TGGCTCAGGA TNAACGCTAG CTATAGGCTT
   1Bi-1   .......... .......... .......... .......... ..........
Pn 33563   .ACAATGGAG AGTTTGATCC TGGCTCAGGA TNAACGCTGG CTACAGGCTT
   2Bi-1   .......... .......... .......... .......... ..........
Pn 25261   .ACAATGGAG AGTTTGATCC TGGCTCAGGA TNAACGCTGG CTACAGGCTT
 RE-TPU1   ........AG AGTTTGATCM TGGCTCAG.. .......... ..........
   P-nig   .......... .......... .......... .......... ..........
   P-int   .......... .......... .......... .......... ..........

           51                                                  100
Pi 25611   AACACATGCA AGTCGAGGGG AAACGGCATT ATGTGCTTGC ACATTCTGGA
   1Bi-1   .......... .......... .......... .......... ..........
Pn 33563   AACACATGCA AGTCGTGGGN NAACGGCATT ATGTGCTTGC ACATTCTGGA
   2Bi-1   .......... .......... .......... .......... ..........
Pn 25261   AACACATGCA AGTCGTGGGN AAACGGCATT ANGTGCTTGC ACATTCTGGA
 RE-TPU1   .......... .......... .......... .......... ..........
   P-nig   .......... .......... .......... .......... ..........
   P-int   .......... .......... .......... .......... ..........

           101                                                 150
Pi 25611   CGTCGACCGG CGCACGGGTG AGTATCGCGN ATCCAACCTT CCCTCCACTC
   1Bi-1   .......... .......... .......... .......... ..........
Pn 33563   CGTTGACCGG CGCACGGGTG AGTATCGCGN ATCCAACCTG CCCCNTACTT
   2Bi-1   .......... .......... .......... .......... ..........
Pn 25261   CGTTGACCGG CGCACGGGTG AGTATCGCGN ATCCAACCTG CCCCNNACTT
 RE-TPU1   .......... .......... .......... .......... ..........
   P-nig   .......... .......... .......... .......... ..........
   P-int   .......... .......... .......... .......... ..........

           151                                                 200
Pi 25611   GGGGATACCC CGTTGAAAGA CGGCCTAATA CCCGATGTTG TCCACATATG
   1Bi-1   .......... .......... .......... .......... ..........
Pn 33563   GGGGATACCC CGTTGAAAGA CGGCCTNATA CCCGATGTGT TTCATTGACG
   2Bi-1   .......... .......... .......... .......... ..........
Pn 25261   GGGGATACCC CGTTGAAAGA CGGCCTNATA CCCGATGTGT TTCATTGACG
 RE-TPU1   .......... .......... .......... .......... ..........
   P-nig   .......... .......... .......... .......... ..........
   P-int   .......... .......... .......... .........TG TCCACATATG

           201                                                 250
Pi 25611   GCATCTGACG TGGACCAAAG ATTCATCGGT GGAGGATGGG GATGCGTCTG
   1Bi-1   .......... .......... .......... .......... ..........
Pn 33563   GCATCCGATA TGAAACAAAG GTTTTCCGGT AAGGGATGGG GATGCGTCTG
   2Bi-1   .......... .......... .......... .......... ..........
Pn 25261   GCATCCGATA TGAAACAAAG GTNTTCCGGT AAGGGATGGG GATGCGTCTG
 RE-TPU1   .......... .......... .......... .......... ..........
   P-nig   .......... .....CAAAG GTTTTCCGGT AAGGGA.... ..........
   P-int   GCATCTGACG TG........ .......... .......... ..........
```

```
            251                                                  300
Pi 25611    ATTAGCTTGT TGGTGCGGGT AACGGCCCAC CAAGGCTNCG ATCAGTAGGG
   1Bi-1    .......... .......... .......... .......... ..........
Pn 33563    ATTAGCTNGT TGGCG.GGGC AACGGCCCAC CAAGGCGACG ATCAGTAGGG
   2Bi-1    .......... .......... .......... .......... ..........
Pn 25261    ATTAGCTTGT TGGCG.GGGC AACGGCCCAC CAAGGCGACG ATCAGTAGGG
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........

            301                                                  350
Pi 25611    GTTCTGAGAG GAAGGTCCCC CACATTGGAA CTGAGACACG GTCCNAACTC
   1Bi-1    .......... .......... .......... .......... ..........
Pn 33563    GTTCTGAGAG GAAGGTCCCC CACATTGGAA CTGAGACACG GTCCNAACTC
   2Bi-1    .......... .......... .......... .......... ..........
Pn 25261    GTTCTGAGAG GAAGGTCCCC CACATTGGAA CTGAGACACG GTCCNAACTC
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........

            351                                                  400
Pi 25611    CTACGGGAGG CAGCAGTGAG GAATATTGGT CAATGGACGT AAGTCTGAAC
   1Bi-1    .......... .......... .......... .......... ..........
Pn 33563    CTACGGGAGG CAGCAGTGAG GAATATTGGT CAATGGACGC AAGTCTGAAC
   2Bi-1    .......... .......... .......... .......... ..........
Pn 25261    CTACGGGAGG CAGCAGTGAG GAATATTGGT CAATGGACGC AAGTCTGAAC
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........

            401                                                  450
Pi 25611    CAGCCAAGTA GCGTGCAGAT TGACGGCCCT ATGGGTTGTA AACTGCTTTT
   1Bi-1    .......... .......... .......... .......... ..........
Pn 33563    CAGCCAAGTA GCGTGCAGGA TGACGGCCCT ATGGGTTGTA AACTNCNTTT
   2Bi-1    .......... .......... .......... .......... ..........
Pn 25261    CAGCCAAGTA GCGTGCAGGA TGACGGCCCT ATGGGTTGTA AACTNCNTTT
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........

            451                                                  500
Pi 25611    GTTGGGGAGT AAAGCGGGCA CGTGTGCCNN NTTGCATTTA CCCTTCGAAT
   1Bi-1    .......... .......... .......... ...GCATTTA CCCTTCGAAT
Pn 33563    ATGTGGGAAT AAA..TTGCA CGTGTGCGCN NTTGCATGTA CCTCATGAAT
   2Bi-1    .......... .......... ..TGTGCGCC ATTGCATGTA CCTCAT....
Pn 25261    ATGTGGGAAT AAA..GTGCA CGTGTGCGCN NTTGCATGTA CCTCATGAAT
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........

            501                                                  550
Pi 25611    AAGGACCGGC TNATTCCGTG CCAGCAGCCG CGGTAATACG GAAGGTCCAG
   1Bi-1    AAGGACC... .......... .......... .......... ..........
Pn 33563    AAGGACCGGC TNATTCCGTG CCAGCAGCCG CGGTAATACG GAAGGTCCNG
   2Bi-1    .......... .......... .......... .......... ..........
Pn 25261    AAGGACCGGC TNATTCCGTG CCAGCAGCCG CGGTAATACG GAAGGTCCNG
 RE-TPU1    .......... .......... .......... .......... ..........
   P-nig    .......... .......... .......... .......... ..........
   P-int    .......... .......... .......... .......... ..........
```

6.13 PAPILLON-LEFÈVRE SYNDROME - PATIENT INFORMATION

Two patients with Papillon-Lefèvre Syndrome attended the University of Manchester Dental Hospital in June 1997.

Patient 1 - E39972

Sex:	male
Date of birth:	01- 01-1993
Age at time of sampling:	4 years 5 months
Ethnicity:	Asian (Saudi Arabia)
Consanguineous parents:	yes
Number of teeth at time of sampling:	6
Sampling sites and order:	upper right deciduous canine
	upper left deciduous canine
	upper left deciduous 1st molar
	lower left deciduous 1st molar
	lower right deciduous canine
	lower right deciduous 1st molar

Patient 2 - D20302

Sex:	female
Date of birth:	17-08-1983
Age at time of sampling	thirteen years 10 months
Ethnicity:	Asian (Pakistan)
Consanguineous parents:	yes
Number of teeth at time of sampling:	11
Sampling sites and order:	upper right permanent 1st molar
	upper right permanent canine
	upper left permanent 1st molar
	lower left permanent 1st molar
	lower right permanent canine
	lower right permanent 1st molar

6.14 COMMERCIAL IDENTIFICATION KITS

For full details regarding the preparation and use of commercial identification kits please see manufacturer's instructions; bioMérieux (API 20 NE, API Coryne, API NH, RapID ID Strep, Rapid ID 32 A) or Innovative Diagnostics Systems (RapID ANA II system). The following tables are adapted from these.

Table 6.4 Reagents and media used with API identification kits (API 20 NE, API Coryne, API NH, Rapid ID 32 Strep)

REAGENT	COMPOSITION
JAMES	0.5% J 2183 (w/v) in hydrochloric acid
NIT I	0.8% sulphanilic acid (w/v) in 5N acetic acid
NIT 2	0.6% N-N-dimethyl-1-naphthylamine(w/v) in 5N acetic acid
FB	0.35% fast blue BB(w/v) in organic solvents
ZYM B	0.35% fast blue BB (w/v) in 2-methoxy-ethanol
ZYM A	25% tri-hydroxy-methyl-amino-methane(w/v); 10% lauryl sulphate(w/v); 11 ml (37%) hydrochloric acid in distilled water
PYZ	1% $FeCl_3$ (w/v), preservative in organic solvent
NIN	7% ninhydrin (w/v) in 2-methoxy ethanol
VP A	20% KOH aq.
VP B	12% alpha naphthol in organic solvent

MEDIA	COMPOSITION
NaCl	0.85% sodium chloride in distilled water
Suspension medium	sterile distilled water
GP medium	0.5% cysteine (w/v); 20% tryptone (w/v); 5% sodium chloride (w/v); 0.5% sodium sulphite (w/v); 0.17% phenol red (w/v) in sterile distilled water
AUX medium	2.0% ammonium sulphate (w/v); 1.5% agar (w/v); 0.0828% mineral base (w/v); 0.25% amino acids (w/v); 0.0359% vitamins/nutritients (w/v) in 0.04M phosphate buffer

Table 6.5 Reagents and media used with RapID ANA II system

REAGENT/MEDIA	COMPOSITION
RapID ANA II reagent	0.01% 3-phenyl 4-methylaminoacrolein; 0.1% hydrochloric acid; 1.0% acetic acid, 0.1% detergent
Innova spot indole reagent	Components not available
RapID inoculation fluid	6.0% potassium chloride (w/v); 0.5% calcium chloride (w/v); 1.6 ml sodium hydroxide (0.1N); in distilled water

6.15 TESTS CONTAINED WITHIN COMMERCIAL IDENTIFICATION KITS

Table 6.6 API 20 NE, substrates and reactions

Adapted from the manufacturer's (bioMérieux) instructions.

TEST CODE	REACTIVE INGREDIENT / SUBSTRATE	REACTION / ENZYME
NO_3	potassium nitrate	reduction of nitrates to nitrites
TRP	tryptophan	substrate utilisation
GLU	glucose	acidification
ADH	arginine	arginine dihydrolase
URE	urea	hydrolysis by urease
ESC	esculin	hydrolysis by β-glucosidase
GEL	gelatine	protease action
PNPG	p-nitro-phenyl-βD-	β-galactosidase
GLU	glucose	assimilation
ARA	arabinose	assimilation
MNE	mannose	assimilation
MAN	mannitol	assimilation
NAG	N-acetyl-glucosamine	assimilation
GNT	gluconate	assimilation
CAP	caprate	assimilation
ADI	adipate	assimilation
MLT	malate	assimilation
CIT	citrate	assimilation
PAC	phenyl-acetate	assimilation
OX	tetramethyl-p-phenylene diamine	cytochrome oxidase

Table 6.7 API Coryne, substrates and reactions

Adapted from the manufacturer's (bioMérieux) instructions.

TEST CODE	REACTIVE INGREDIENT / SUBSTRATE	REACTION / ENZYME
NIT	potassium nitrate	reduction of nitrates
PYZ		pyrazinamidase
PyrA	pyrolidonyl	pyrrolidonyl arylamidase
PAL		alkaline phosphatase
β GUR		β glucuronidase
β GAL	galactoside	β galactosidase
α GLU	glucoside	α glucosidase
β NAG	N-acetyl-β glucosamine	N-acetyl-β glucosaminidase
ESC	esculin	hydrolysis by β-glucosidase
URE	urea	hydrolysis by urease
GEL	gelatine	protease action (hydrolysis)
GLU	glucose	fermentation
RIB	ribose	fermentation
XYL	xylose	fermentation
MAN	mannitol	fermentation
LAC	lactose	fermentation
SAC	sucrose	fermentation
GLYG	glycogen	fermentation
CAT	catalase	hydrogen-peroxide breakdown

Table 6.8 API NH, reactions and enzymes

Adapted from the manufacturer's (bioMérieux) instructions.

TEST CODE	REACTION / ENZYME
PEN	penicillinase
GLU	acidification of glucose
FRU	acidification of fructose
MAL	acidification of maltose
SAC	acidification of sucrose
ODC	ornithin decarboxylase
URE	urease
LIP	lipase
PAL	alcaline phosphatase
βGAL	β galactosidase
ProA	proline arylamidase
GGT	gamma glutamyl transferase
IND	indole production

Table 6.9 RapID ANA II system , substrates and reactions

Adapted from the manufacturer's (Innovative Diagnostic Systems) instructions.

TEST CODE	REACTIVE INGREDIENT / SUBSTRATE	REACTION / ENZYME
URE	urea	hydrolysis by urease
BLTS	p-Nitrophenyl-B, D-disaccharide	hydrolysis of glycoside
αARA	p-Nitrophenyl-a, L-arabinoside	hydrolysis of glycoside
ONPG	o-Nitrophenyl-B, D-galactoside	hydrolysis of glycoside
αGLU	p-Nitrophenyl-a, D-glucoside	hydrolysis of glycoside
βGLU	p-Nitrophenyl-B, D-glucoside	hydrolysis of glycoside
αGAL	p-Nitrophenyl-a, D-galactoside	hydrolysis of glycoside
αFUC	p-Nitrophenyl-a, L-fucoside	hydrolysis of glycoside
NAG	p-Nitrophenyl-n-acetyl-B, D-glucosaminide	hydrolysis of glycoside
PO4	p-nitrophenylphosphate	hydrolysis of phosphester
LGY	leucyl-glycine-B-naphthylamide	hydrolysis of arylamide
GLY	glycine-B-naphthylamide	hydrolysis of arylamide
PRO	proline-B-naphthylamide	hydrolysis of arylamide
PAL	phenylalanine-B-naphthylamide	hydrolysis of arylamide
ARG	arginine-B-naphthylamide	hydrolysis of arylamide
SER	serine-B-naphthylamide	hydrolysis of arylamide
PYR	pyrolidonyl-B-naphthylamide	hydrolysis of arylamide
IND	tryptophan	substrate utilisation

Table 6.10 Rapid ID 32 Strep, enzymes and reactions

Adapted from the manufacturer's (bioMérieux) instructions.

TEST CODE	ENZYME / REACTION
ADH	arginine dihydrolase
βGLU	β glucosidase
βGAR	β galactosidase
βGUR	β glucuronidase
αGAL	α galactosidase
PAL	alcaline phosphatase
RIB	acidification of ribose
MAN	acidification of mannitol
SOR	acidification of sorbitol
LAC	acidification of lactose
TRE	acidification of trehalose
RAF	acidification of raffinose
SAC	acidification of sucrose
LARA	acidification of L-arabinose
DARL	acidification of D-arabitol
CDEX	acidification of cyclodextrin

TEST CODE	ENZYME / REACTION
VP	acetoin production
APPA	alanine-phenylalanine-proline arylamidase
βGAL	β galactosidase
PyrA	pyroglutamic acid arylamidase
βNAG	N-acetyl-β glucosaminidase
GTA	glycyl-tryptophane arylamidase
HIP	hydrolysis of hippurate
GLYG	acidification of glycogen
PUL	acidification of pullulan
MAL	acidification of maltose
MEL	acidification of melibiose
MLZ	acidification of melezitose
MBDG	acidification of methyl-B-D glucopyranoside
TAG	acidification of tagatose
βMAN	β mannosidase
URE	hydrolysis by urease

Table 6.11 Rapid ID 32 A, enzymes and reactions

Adapted from the manufacturer's (bioMérieux) instructions.

TEST CODE	ENZYME / REACTION
URE	urease
ADH	arginine dihydrolase
αGAL	α galactosidase
βGAL	β galactosidase
βGP	βgalactosidase 6 phosphate
αGLU	α glucosidase
βGLU	β glucosidase
αARA	α arabinosidase
βGUR	β glucuronidase
βNAG	β N-acetyl-glucosamininidase
MNE	mannose fermentation
RAF	raffinose fermentation
GDC	glutamic acid decarboxylase
αFUC	α fucosidase
NIT	reduction of nitrates

TEST CODE	ENZYME / REACTION
IND	indole production
PAL	alcaline phosphatase
ArgA	arginine arylamidase
ProA	proline arylamidase
LGA	leucyl glycine arylamidase
PheA	phenylalanine arylamidase
LeuA	leucine arylamidase
PyrA	pyroglutamic acid arylamidase
TyrA	tyrosine arylamidase
AlaA	alanine arylamidase
GlyA	glycine arylamidase
HisA	histidine arylamidase
GGA	glutamyl glutamic acid arylamidase
SerA	serine arylamidase

6.16 REAGENTS AND MEDIUM USED FOR TRADITIONAL IDENTIFICATION TESTS

6.16.1 HYDROGEN PEROXIDE

A 3% aqueous solution of hydrogen peroxide was required for the catalase test.

6.16.2 OXIDASE REAGENT

The colourless oxidase reagent, 1% tetramethyl - p- phenylenediamine aqueous solution was prepared as described by Cowan and Steel (1993). It was protected from light and stored at 4°C. This reagent was not used once it had turned blue.

6.16.3 HUGH AND LEIFSON'S OXIDATION-FERMENTATION MEDIUM

The following components were dissolved by heating in 1 litre of distilled water; peptone 29 g; sodium chloride 5 g; K_2HPO_4 0.3 g and agar 3 g. The pH was adjusted to 7.1 and bromothymol blue 0.2% aq. sol. 15 ml added. Sterilisation was by autoclaving at 121°C (15 lb/in^2, 1.05 bar; Cowan and Steel, 1993) for 15 min. Glucose (1%) was added and the media distributed into 10 ml aliquots in sterile universals.

6.16.4 NITRATE REDUCTION TEST REAGENTS

Two reagents were required. Solution A; 0.33% sulphanilic acid was dissolved with gentle heating in 5 N-acetic acid. Solution B; 0.6% dimethyl-α-naphthylamine was dissolved with gentle heating in 5 N-acetic acid.

6.17 RESULT PROFILES FOR PLS ISOLATES SUCCESSFULLY IDENTIFIED USING COMMERCIAL IDENTIFICATION KITS.

6.17.1 ISOLATES LABELLED AS AEROBIC

Table 6.12 Individual test results for aerobic and facultative isolates successfully identified to at least genus level using Rapid ID 32 Strep identification kit

	4 *1F*	2 *2A*	3 *1F*	3 *1A*	1 *1C*	9 *1B*
ADH	-	-	-	-	-	-
βGLU	-	+	-	-	-	-
βGAR	-	+	-	+	+	+
βGUR	-	-	-	-	-	-
αGAL	-	+	-	-	-	-
PAL	+	-	-	-	-	+
RIB	-	-	-	-	-	-
MAN	-	-	-	-	-	-
SOR	-	-	-	-	-	-
LAC	-	-	+	+	+	+
TRE	+	+	+	-	+	-
RAF	-	+	-	+	+	+
SAC	+	+	+	+	+	+
LARA	-	-	-	-	-	-
DARL	-	-	-	-	-	-
CDEX	-	-	-	-	-	-
VP	+	-	-	-	+	-
APPA	+	+	+	+	+	+
βGAL	-	-	-	-	-	-
PyrA	-	-	-	-	-	-
βNAG	-	-	-	-	-	-
GTA	-	-	-	-	-	+
HIP	-	-	-	-	+	-
GLYG	-	-	-	-	-	-
PUL	+	+	+	+	-	+
MAL	+	+	+	+	+	+
MEL	-	-	-	-	-	-
MLZ	-	-	-	-	-	-
MBDG	-	+	-	-	-	-
TAG	-	-	-	-	-	-
βMAN	-	-	-	-	-	-
URE	+	+	-	-	-	-
Code	0402 3041 102	6207 2041 112	0003 2041 100	4005 2041 100	2207 3041 100	4405 2441 100
ID	*S. saliv. salivarius*	*S. mitis*	*Strep.* sp. *S. mitis*	*S. bovis* II	*S. oralis*	

Table 6.12 Individual test results for aerobic and facultative isolates successfully identified to at least genus level using Rapid ID 32 Strep (continued)

	11 *2A*	**4** *1B*	**12** *1A*	**8** *1B*	**2** *1D*	**3** *1D*
ADH	+	+	+	-	-	-
βGLU	+	-	-	+	-	-
βGAR	-	-	-	-	+	-
βGUR	-	-	-	-	-	-
αGAL	-	-	+	-	-	-
PAL	+	+	+	-	-	+
RIB	-	-	-	-	-	-
MAN	-	-	-	-	-	-
SOR	-	-	-	-	-	-
LAC	-	+	-	+	-	-
TRE	-	+	+	+	+	-
RAF	-	-	-	+	-	-
SAC	+	+	-	+	+	-
LARA	-	-	-	-	-	-
DARL	-	-	-	-	-	-
CDEX	-	-	-	-	-	-
VP	+	+	+	-	-	-
APPA	+	+	+	+	+	-
βGAL	-	-	-	-	-	-
PyrA	-	-	-	-	-	-
βNAG	-	-	-	-	-	-
GTA	-	-	-	-	+	-
HIP	-	-	-	-	-	-
GLYG	-	-	-	-	-	-
PUL	-	-	-	+	-	-
MAL	+	+	-	+	+	-
MEL	-	-	-	-	-	-
MLZ	-	-	-	-	-	-
MBDG	+	+	-	-	-	-
TAG	-	-	-	-	-	-
βMAN	-	-	-	-	-	-
URE	-	-	-	-	+	-
Code	3400 300 110	1403 3001 110	1602 3000 00	2007 2041 100	4002 2401 102	0400 0000 000
ID	*S. constellatus*			*S. sanguis*	*Gemella morbill-orum*	*G. haemoly-sans*

KEY: for test codes see table 6.10.

Table 6.13 Individual test results for aerobic and facultative isolates successfully identified to genus level using API NH identification kit

	7 *1B*	5 *1C*	6 *1A*
PEN	-	-	-
GLU	+	+	+
FRU	+	+	+
MAL	+	+	-
SAC	+	-	-
ODC	-	-	-
URE	-	-	-
LIP	-	-	-
PAL	-	-	-
βGAL	-	-	-
ProA	+	+	+
GGT	-	-	-
IND	-	-	-
Code	7101	7001	3001
ID	*Neisseria* sp.		

KEY: for test codes see table 6.8.

Table 6.14 Individual test results for aerobic and facultative isolates successfully identified to genus level using RapID ANA II system

	2 *1A*	10 *1A*	8 *1A*	2 *1B*
URE	-	-	-	-
BLTS	+	-	+	-
αARA	-	-	-	-
ONPG	+	-	-	-
αGLU	+	+	+	+
βGLU	+	-	+	-
αGAL	-	-	-	+
αFUC	-	-	-	-
NAG	-	+	+	-
PO4	+	+	+	-
LGY	+	+	+	-
GLY	+	+	+	-
PRO	+	+	+	+
PAL	+	+	+	+
ARG	+	+	+	-
SER	+	+	+	-
PYR	-	-	-	-
IND	-	-	-	-
Code	270771	224791	264771	021030
ID	*Capnocytophaga* sp.			*Mobil-uncus* sp.

KEY: for test codes see table 6.9.

6.17.2 ISOLATES LABELLED AS ANAEROBIC AND FACULATATIVE

Table 6.15 Individual test results for anaerobic and facultative isolates successfully identified using Rapid ID 32 A

	5 *1D*	1 *2B*	3 *2A*	18 *1D*	8 *1A*	5 *2B*	17 *2A*	6 *2A*
URE	-	-	-	-	-	-	-	-
ADH	-	-	-	+	-	-	-	-
αGAL	-	-	-	-	-	+	+	+
βGAL	-	-	-	+	+	+	+	+
βGP	-	-	-	-	+	+	+	+
αGLU	+	+	+	-	+	+	+	+
βGLU	-	-	-	+	-	-	-	-
αARA	-	-	-	-	-	-	-	-
βGUR	-	-	-	-	-	-	-	-
βNAG	-	-	-	+	+	+	+	+
MNE	-	+	+	+	-	-	+	-
RAF	-	+	+	-	-	-	+	+
GDC	-	-	-	-	-	+	-	-
FUC	+	-	+	-	+	+	+	+
NIT	-	-	-	-	-	-	-	-
IND	+	-	+	-	-	-	-	-
PAL	+	-	+	-	+	+	+	+
ArgA	-	+	+	+	+	+	+	+
ProA	-	-	-	-	-	-	-	-
LGA	+	+	+	-	+	+	+	+
PheA	-	-	-	+	-	-	-	-
LeuA	-	-	-	+	-	-	-	-
PyrA	-	-	-	-	-	-	-	-
TyrA	-	-	-	+	-	-	-	-
AlaA	+	+	+	+	+	+	+	+
GlyA	-	-	-	-	-	-	-	-
HisA	-	-	-	+	-	-	-	-
GGA	-	+	-	-	-	-	+	+
SerA	-	-	-	-	-	-	-	-
Code	0404 6402 20	0406 6502 22	0406 6502 22	2113 0133 01	0701 4502 20	4701 4502 30	4707 4502 22	4705 4502 22
ID	*P. intermedia*			*G. morbil-lorum*	*Prevo-tella* sp.	*P. melaninogenica*		

KEY: for test codes see table 6.11.

Table 6.16 Individual test results for anaerobic and facultative isolates successfully identified using RapID ANA II system

	1 *2B*	**5** *1D* **3** *2A*	**18** *2A*	**2** *1B*	**3** *1D*
URE	-	-	-	-	-
BLTS	-	-	-	-	-
αARA	-	-	-	-	-
ONPG	-	-	-	-	-
αGLU	+	+	-	+	-
βGLU	-	-	-	+	-
αGAL	-	-	-	+	-
αFUC	+	+	-	-	-
NAG	-	-	-	-	-
PO4	+	+	-	-	+
LGY	+	+	-	-	+
GLY	-	-	-	-	-
PRO	-	-	+	+	+
PAL	-	-	-	+	+
ARG	-	+	-	+	+
SER	-	-	-	+	+
PYR	-	-	-	-	+
IND	+	+	-	-	-
Code	022304	022344	000010	021030	000373
ID	*P. intermedia*		*Ps. anaerob-ius*	*Mobilun-cus* sp.	*Ps. micros*

KEY: for test codes see table 6.9.

Table 6.17 Individual test results for anaerobic and facultative isolates successfully identified by the Rapid ID 32 Strep kit

	2 *2A*
ADH	-
βGLU	+
βGAR	+
βGUR	-
αGAL	+
PAL	-
RIB	-
MAN	-
SOR	-
LAC	-
TRE	-
RAF	-
SAC	-
LARA	-
DARL	-
CDEX	-
VP	-
APPA	+
βGAL	-
PyrA	-
βNAG	-
GTA	-
HIP	-
GLYG	-
PUL	-
MAL	-
MEL	-
MLZ	-
MBDG	-
TAG	-
βMAN	-
URE	-
Code	6200 2000 000
ID	*Leuconostoc* spp.

KEY:

For test codes see table 6.10.

6.18 AEROBIC, ANAEROBIC AND FACULTATIVE PLS ISOLATES WHICH CEASED TO BE VIABLE BEFORE IDENTIFICATION

The following two tables detail those isolates which died before identifications could be reached and therefore no identification is made as well as those for which identities could not be confirmed by other tests.

Table 6.18 Clinical isolates which ceased to be viable before identification was carried out or completed

AEROBIC (AND FACULTATIVE) ISOLATES
1 *1E* *
1 *1F* *
2 *1A* +
2 *1C* +
2 *1F* *
3 *1B* *
3 *2B* *
4 *1A* +
4 *1B* +
4 *2A* +
5 *1A* *
6 *2A* *
8 *1A* +
8 *1B* +
8 *2A* *
9 *2F* *
10 *1A* *
11 *1B* *

ANAEROBIC (AND FACULTATIVE) ISOLATES
3 *1B* *
3 *1E* *
3 *2B* *
4 *1E* *
4 *2A* *
4 *2B* *
5 *1B* *
7 *1A* *
7 *1B* *
10 *1B* *
10 *2A* *
10 *2B* *
11 *2A* *
13 *2A* *
14 *2E* *

KEY:

* non-viable before any identification could be made; no identification given.

+ non-viable before identification could be completed or verified by tests other than identification kits; identification given.

6.19 RETENTION TIMES FROM GLC ANALYSIS

Table 6.19 Range of retention times of volatile acids and alcohols eluted from the column and corresponding median value

VOLATILE ACID / ALCOHOL	RETENTION TIME RANGE (MIN)	MEDIAN VALUE (MIN)
Ethanol	0.14 - 0.18	0.17
Propanol	0.17 - 0.24	0.215
Butanol	0.55 - 1.10	1.03
Acetic	2.43 - 3.06	2.545
Propionic	3.17 -3.39	3.25
Iso-butyric	3.23 - 3.51	3.32
Butyric	4.0 - 4.30	4.09
Iso-valeric	4.16 - 4.58	4.27
Valeric (Pentanoic)	5.15 - 6.05	5.295

Table 6.10 Range of retention times of non-volatile acids eluted from the column and corresponding median value

NON -VOLATILE ACID	RETENTION TIME RANGE (MIN)	MEDIAN VALUE (MIN)
Lactic	1.56 - 2.13	2.08
Oxalic	2.24 - 2.40	2.345
Malonic	3.01 - 3.24	3.15
Succinic	3.36 - 4.04	3.55

6.20 DIFFERENTIATION OF GENERA OF ANAEROBES BY GLC ANALYSIS OF FERMENTATION END PRODUCTS

Table 6.21 Genus level identifications by GLC of fermentation end-products

GRAM-NEGATIVE BACILLI	
Non-motile or peritrichous flagella	
Produce butyric acid	*Fusobacterium*
Produce major lactic acid	*Leptotrichia*
Produce acetic acid	*Desulfomonas*
Not as above	*Bacteroides*
	Prevotella
	Porphyromonas
	Bilophila
Motile	
Fermentative	
Produce acetic acid	*Mobiluncus*
Non-fermentative	
Produce succinic acid from fumarate	*Campylobacter*
GRAM-NEGATIVE COCCI	
Produce propionic and acetic acids	*Veillonella*
GRAM-POSITIVE COCCI	
Lactic acid sole major product	*Streptococcus*
	Gemella
Not as above	*Peptostreptococcus*
	Peptococcus
GRAM-POSITIVE BACILLI	
Spore forming	*Clostridium*
Non-sporeforming	
Produce propionic and acetic acids	*Propionibacterium*
No propionic acid produced	
Produce acetic and lactic acid ($A \geq L$)	*Bifidobacterium*
Lactic acid sole major product	*Lactobacillus*
Moderate acetic acid plus succinic and / or lactic acids	*Actinomyces*
Not as above, butyric $\pm$ others, acetic or no major acids	*Eubacterium*

Adapted from the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993). Only those parts of the table which reflect the identification of oral bacteria have been chosen for this adaptation.

6.21 PARTIAL 16S rDNA SEQUENCES - PLS ISOLATES

Following extraction of genomic DNA, a region of the 16S rRNA gene was amplified with universal primers RE-TPU1 and RE-RTU3 and subjected to cycle sequencing reactions (2.6.1-2.6.7). The sequences were manipulated as described previously (section 2.6.8, appendix 6.8). The closest phylogenetic relationships (determined by FASTA search) and 16S rDNA sequences are given below. International base codes are listed in appendix 6.5.

6.21.1 AEROBIC AND FACULTATIVE ISOLATES

Isolate **1** *1D* : *S. sanguis*: 98.0% identity in 456 bp overlap (sequence length 456 bp)

```
  1 TACATGCAAG TAGAACGCTG AAGAGAGGAG CTTGCTCTTC TTGGATGAGT
 51 TGCGAACGGG TGAGTAACGC GTAGGTAACC TGCCTGGTAG CGGGGGATAA
101 CTATTGGAAA CGATAGCTAA TACCGCATAA AATTGATTAT CGCATGATAA
151 TTAATTGAAA GGTGCAAATG CATCACTACC AGATGGACCT GCGTTGTATT
201 AGCTAGTTGG TGGGGTAACG GCTCACCAAG GCGACGATAC ATAGCCGACC
251 TGAGAGGGTG ATCGGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
301 GGGAGGCAGC AGTAGGGAAT CTTCGGCAAT GGACGGAAGT CTGACCGAGC
351 AACGCCGCGT GAGTGAAGAA GGTTTTCGGA TCGTAAAGCT CTGTTGTAAG
401 AGAAGAACGA GTGTGAGAGT GGAAAGTTCA CACTGTGACG GTATCTTACC
451 AGAAAG
```

Isolate **1** *2B* : *S. gordonii*: 99.4% in 467 bp overlap (sequence length 467 bp)

```
  1 GTGCCTAATA CATGCAAGTA GAACGCACAG TTTATACCGT AGCTTGCTAC
 51 ACCATAGACT GTGAGTTGCG AACGGGTGAG TAACGCGTAG GTAACCTGCC
101 TGGTAGCGGG GGATAACTAT TGGAAACGAT AGCTAATACC GCATAATATT
151 AATTATTGCA TGATAATTGA TTGAAAGATG CAATTGCATC ACTACCAGAT
201 GGACCTGCGT TGTATTAGCT AGTAGGTGAG GTAACGGCTC ACCTAGGCGA
251 CGATACATAG CCGACCTGAG AGGGTGATCG GCCACACTGG GACTGAGACA
301 CGGCCCAGAC TCCTACGGGA GGCAGCAGTA GGGAATCTTC GGCAATGGAC
351 GAAAGTCTGA CCGAGCAACG CCGCGTGAGT GAAGAAGGTT TTCGGATCGT
401 AAAGCTCTGT TGTAAGAGAA GAACGGGTGT GAGAGTGGAA AGTTCACACT
451 GTGACGGTAT CTTACCA
```

Isolate **3** *1C* : *S. sanguis*: 97.2% in 431 bp overlap (sequence length 431 bp)

```
  1 CAAGTAGAAC GCTGAAGAGA GGAGCTTGCT CTTCTTGGAT GAGTTGCGAA
 51 CGGGTGAGTA ACGCGTAGGT AACCTGCCTG GTAGCGGGGG ATAACTATTG
101 GAAACGATAG CTAATACCGC ATAAAATGGA TTATCGCATG ATRATTCATT
151 GAAAGGTGCA AATGCATCAC TACCAGATGG ACCTGCGTTG TATTAGCTAG
201 TTGGTGGGGT AACGGCTCAC CAAGGCGACG ATACATAGCC GACCTGAGAG
251 GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG
301 CAGCAGTAGG GAATCTTCGG CAATGGACGG AAGTCTGACC GAGCAACGCC
351 GCGTGAGTGA AGAAGGTTTT CGGATCGTAA AGCTCTGTTG TAAGAGAAGA
401 ACGAGTGTGA GAGTGGAAAG TTCACACTGT G
```

Isolate **3** *1E* : *S. indologenes*: 86.1% in 452 bp overlap (sequence length 452 bp)

```
  1 ATGCTTAACA CATGCAAGTC GAACGGAAAC GATGGAGCTT GCTCCAGGCG
 51 TCGAGTGGCG GACGGGTGAG TAACGCATGG GAATCTGCCT TTTGCTGGGG
101 GATAACGTAG GGAAACTTAC GCTAATACCG CATAAGACCT AAGGGTGAAA
151 GCGGGGGACC GAAAGGCCTC GCGGCAAGAG ATGAGCCCAT GTTGGATTAG
```

```
201 CTAGTTGGTG GGGTAAAGGC CTACCAAGGC GACGATCCAT AGCTGGTCTG
251 AGAGGATGAT CAGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
301 GAGGCAGCAG TGGGGAATAT TGGACAATGG GGGGAACCCT GATCCAGCAA
351 TGCCGCGTGT GTGAAGAAGG CCTTCGGGTT GTAAAGCACT TTCAGTAGGG
401 AGGAAAGGTG CGTAGTTAAT ACCTGCGCAA TTGACGTTAC CTACAGAAGA
451 AG
```

Isolate **3** *2A* : *N. elongata*: 99.4 % in 470 bp overlap (sequence length 470 bp)

```
  1 CTGGCGGCAT GCTTTACACA TGCAAGTCGG ACGGCAGCGG GGTAGTGCTT
 51 GCACTACTGC CGGCGAGTGG CGAACGGGTG AGTAATATAT TGGAACGTAC
101 CGAGTAATGG GGGATAACCA ATCGAAAGAT TGGCTAATAC CGCATACGCT
151 CTGAGGAGGA AAGCAGGGGA CCTTCGGGCC TTGCGTTATT CGAGCGGCCA
201 ATATCTGATT AGCTAGTTGG TGGGGTAAAG GCCTACCAAG GCGACGATCA
251 GTAGCGGGTC TGAGAGGATG ATCCGCCACA CTGGGACTGA GACACGGCCC
301 AGACTCCTAC GGGAGGCAGC AGTGGGGAAT TTTGGACAAT GGGCGCAAGC
351 CTGATCCAGC CATGCCGCGT GTCTGAAGAA GGCCTTCGGG TTGTAAAGGA
401 CTTTTGTTAG GGAAGAAAAA GGAGCGGTTA ATACCCGTTT CTGCTGACGG
451 TACCTAAAGA ATAAGCACCG
```

Isolate **4** *1C II* : *Stom. mucilaginosus*: 99.8% in 440 bp overlap (sequence length 440 bp)

```
  1 CGTGCTTAAC ACATGCAAGT CGAACGATGA AGCCTAGCTT GCTAGGTGGA
 51 TTAGTGGCGA ACGGGTGAGT AATACGTGAG TAACCTACCT TTAACTCTGG
101 GATAAGCcTG GGAAACTGGG TCTAATACCG GATACGACCA ATCTCCGCAT
151 GGGGTGTTGG TGGAAAGCGT TATGTAGTGG TTATAGATGG GCTCACGGCC
201 TATCAGCTTG TTGGTGAGGT AACGGCTCAC CAAGGCGACG ACGGGTAGCC
251 GGCCTGAGAG GGTGACCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
301 CTACGGGAGG CAGCAGTGGG GAATATTGCA CAATGGGCGC AAGCCTGATG
351 CAGCGACGCC GCGTGAGGGA TGACGGCCTT CGGGTTGTAA ACCTCTGTTA
401 GCAGGGAAGA AGAGAGATTG ACGGTACCTG CAGAGAAAGC
```

Isolate **5** *2A* : *S. anginosus*: 97.6% in bp overlap (sequence length 480 bp)

```
  1  AACKCTGGCG GCGTGCCTAA TACATGCAAG TAGGACGCAC AGTCTATACC
 51  GTAGCTTGCT ACACCATAGG CTGTGAGTTG CGAACGGGWG AGTAACGCGT
101  AGGTAACCTG CCTATTAGAG GGGGATAACT ATTGGAAACG ATAGCTAATA
151  CCGCATAACA GTATGTAACA CATGTTAGAT GCTTGAAAGA TGCAATTGCW
201  TCGCTAGTAG ATGGACCTGC GTTGTATTAG CTAGTAGGTA GGGTAATGGC
251  CTACCTAGGC GACGATACAT AGCCGACCYK RAGAGGGTGA DCGGCCACAC
301  TGGGACTGAG ACACGGCCCA GACKCCTACG GGAGGCAGCA GTAGGGAATC
351  TTCGGCAATG GGGGGAACCC TGACCGAGCA ACGCCGCGTG AGTGAAGAAG
401  GTTTTCGGAC CGTAAAGCYC TGTTGTTAAG GAAGAACGAG TGTGAGAATG
451  GAAAGTTCAT ACTGTGACGG TACTTAACCA
```

Isolate **6** *2B* : *S. milleri* group: 85.6% in 439 bp overlap (sequence length 440 bp)

```
  1 TAATACATGC AAGTASAACG CACAGGATGC MCCGtAgTTT HCTACACCGT
 51 ATTCTGTGAG TTGCGAaCGG gTGAGTaACG CGTaGGTAAC CTGCCTGGYA
101 KCGGGGgATA ACTATTGGAM SCGATWGCTA ATACCGSATA AGARCATTTR
151 YYGcATGGTM SATGTTYAAA AGSTGCAAAT SCATCASTAC CAGATGGACC
201 TGMRTTGTAT TAGCTACTAG GTKAVGTAAC GGCTCMCCTA GGCGACSATW
251 CATAACCKAC YTKARAGGGT GATCGVCCMC MCTGGgACTK ARACMCGGMC
301 CAGACTCCTA CgGKAGGCAR CVGTaGGGAA TCTTCgGCMA TGGgGGGAAC
351 CCTKACCGAR CMVCKCCGSG TGAGTGAAGA ARGTTTTCGG ATCGTAAAGC
401 TCYGTTGATA AGGAAKAACG AGTGTTAGAA TGRHMAGTTC
```

Isolate **9** *2A* : *N. flavescens*: 98.0% in 457 bp overlap (sequence length 457 bp)

```
  1 TGCTTTACAC ATGCAAGTCG GACGGCAGCA CAGAGAAGCT TGCTTCTTGG
 51 GTGGCGAGTG GCGAACGGGT GAGTAACATA TCGGAACGTA CCGAGTAATG
101 GGGGATAACT AATCGAAAGA TTAGCTAATA CCGCATATTC TCTGAGGAGG
151 AAAGCAGGGG ACCTTCGGGC CTTGCGTTAT TTGAGCGGCC GATATCTGAT
```

```
201 TAGCTAGTTG GTGGGGTAAA GGCCTACCAA GGCGACGATC AGTAGCGGGT
251 CTGAGAGGAT GATCCGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA
301 CGGGAGGCAG CAGTGGGGAA TTTTGGACAA TGGGCGCAAG CCTGATCCAG
351 CCATGCCGCG TGTCTGAAGA AGGCCTTCGG GTTGTAAAGG ACTTTTGTCA
401 GGGAAGAAAA GGCTGTTGCT AATATCGACA GCTGATGACG GTACCTGAAG
451 AATAAGC
```

Isolate **9** *2E* : *L. mesenteroides* / *L. cremoris*: 99.3% in 458 bp overlap (sequence length 458 bp)

```
  1 ATACATGCAA GTCGAACGCA CAGCGAAAGG TGCTTGCaCC TTTCAAGTGA
 51 GTGGCGAACG GGTGAGTAAC ACGTGGACAA CCTGCCTCAA GGCTGGGGAT
101 AACATTTGGA AACAGATGCT AATACCGAAT AAAACTTAGT GTCGCATGAC
151 ACAAAGTTAA AAGGCGCTTC GGCGTCACCT AGAGATGGAT CCGCGGTGCA
201 TTAGTTAGTT GGTGGGGTAA AGGCCTACCA AGACAATGAT GCATAGCCGA
251 GTTGAGAGAC TGATCGGCCA CATTGGGACT GAGACACGGC CCAAACTCCT
301 ACGGGAGGCT GCAGTAGGGA ATCTTCCACA ATGGGCGAAA GCCTGATGGA
351 GCAACGCCGC GTGTGTGATG AAGGCTTTCG GGTCGTAAAG CACTGTTGTA
401 TGGGAAGAAC AGMTAGAATA GGAAATGATT TTAGTTTGAC GGTACCATAC
451 CAGAAAGG
```

Isolate **10** *2A* : *S. sanguis*: 96.6% identity in 464 bp overlap (sequence length 464 bp)

```
  1 GTGCCTAATA CATGcaAGTA GAACGCTGaa GAGAGGAGCT TGCTCTTCTT
 51 GGATGAGTTG CGAACGGGTG AGTAACGCGT AGGTAACCTG CCTGGTAGCG
101 GGGGATaaCT ATTGGAAACG ATAGCTAATA CCGCATAATA GCAGTTATTG
151 CATGATAACT GTTTGAAAGG TGCAATTGCA CCACTACCAG ATGGACCTGC
201 GTTGTATTAG CTAGTTGGTG GGGTAACGGC TCACCAAGGC GACGATACAT
251 AGCCGACCTG AGAGGGTGAT CGGCCACACT GGGACTGAGA CACGGCCCAG
301 ACTCCTACGG GAGGCAGCAG TAGGGAATCT TCGGCAATGG ACGGAAGTCT
351 GACCGAGCAA CGCCGCGTGA GTGAAGAAGG TTTTCGGATC GTAAAGCTCT
401 GTTGTAAGAG AAGAACGAGT GTGAGAGTGG AAAGTTCACA CTGTGACGGT
451 ATCTTACCAG AAAG
```

Isolate **11** *2A* : *S. anginosus*: 99.6% in 454 bp overlap (sequence length 455 bp)

```
  1 CAAGTAGGAC GCACAGTCTA TACCGTAGCT TGCTACACCA TAGGCTGTGA
 51 GTTGCGAACG GGTGAGTAAC GCGTAGGTAA CCTGCCTATT AGAGGGGGAT
101 AACTATTGGA AACGATAGCT AATACCGCAT AACAGTATGT AACACATGTT
151 AGATGCTTGA AAGATGCAAT TGCATCGCTA GTAGATGGAC CTGCGTTGTA
201 TTAGCTAGTA GGTAGGGTAA TGGCCTACCT AGGCGACGAT ACATAGCCGA
251 CCTGAGAGGG TGATCGGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
301 ACGGGAGGCA GCAGTAGGGA ATCTTCGGCA ATGGGGGGAA CCCTGACCGA
351 GCAACGCCGC GTGAGTGAAG AAGGTTTTCG GATCGTAAAG CTCTGTTGTT
401 AAGGAAGAAC GAGTGTGAGA ATGGAAAGTT CATACTGTGA CGGTACTTAA
451 CCAGA
```

Isolate **14** *2A* : *N. flavescens*: 98.0% in 458 bp overlap (sequence length 458 bp)

```
  1 ATGCTTTACA CATGCAAGTC GGACGGCAGC ACAGAGAAGC TTGCTTCTTG
 51 GGTGGCGAGT GGCGAACGGG TGAGTAACAT ATCGGAACGT ACCGAGTAAT
101 GGGGGATAAC TAATCGAAAG ATTAGCTAAT ACCGCATATT CTCTGAGGAG
151 GAAAGCAGGG GACCTTCGGG CCTTGCGTTA TTTGAGCGGC CGATATCTGA
201 TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CAGTAGCGGG
251 TCTGAGAGGA TGATCCGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
301 ACGGGAGGCA GCAGTGGGGA ATTTTGGACA ATGGGCGCAA GCCTGATCCA
351 GCCATGCCGC GTGTCTGAAG AAGGCCTTCG GGTTGTAAAG GACTTTTGTC
401 AGGGAAGAAA AGGCTGTTGC TAATATCGAC AGCTGATGAC GGTACCTGAA
451 GAATAAGC
```

Isolate **16** *1A* : *Kingella denitrificans*: 98.6% in 438 bp overlap (sequence length 438 bp)

```
  1 AAGTCGGACG GCAGCGGAGG TGCTTGCACC TGCCGGCGAG TGGCGAACGG
 51 GTGAGTAACA TATTGGAATG TACCGAGTAG TGGGGGATAA CCAATCGAAA
101 GATTGGCTAA TACCGCATAC GTCCTGAGGG GGAAAGCAGG GGATCTTCGG
151 ACCTTGTGCT ATTTGAGCAG CCAATATCTG ATTAGCTAGT TGGTGGGGTA
201 AGGGCCTACC AAGGCGACGA TCAGTAGCGG GTCTGAGAGG ATGATCCGCC
251 ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC AGCAGTGGGG
301 AATTTTGGAC AATGGGGGCA ACCCTGATCC AGCCATGCCG CGTGTCTGAA
351 GAAGGCCTTC GGGTTGTAAA GGACTTTTGT TAGGGAAGAA AAGAGAAGTG
401 TTAATACCAC TTTTTGCTGA CGGTACCTAA AGAATAAG
```

6.21.2 ANAEROBIC AND FACULTATIVE ISOLATES

Isolate **1** *1B* : *A. israelii*: 98.0% in 455 bp overlap (sequence length 466 bp)

```
  1 GTGCTTAACA CATGCAAGTC GAACGGGTCT GCCTTGTTTT TTGCGGGGTG
 51 GGTCAGTGGC GAACGGGTGA GTAACACGTG AGTAACCTGC CCCTCACTTC
101 TGGATAACCG CTTGAAAGGG TGGCTAATAC GGGGTGTTCT GGCTGTGCCG
151 CATGGTGTGG CTGGGAAAGA TTCACTTTTG TGGTGTTTTG GTGGGGGATG
201 GGCTCGCGGC CTATCAGCTT GTTGGTGGGG TGATGGCCTG CCAAGGCTTT
251 GACGGGTAGC CGGCCTGAGA GGGTGGGCGG TCACACTGGG ACTGAGATAC
301 GGMCCAGACT CCTGCRGGAG GCAGCAGTGG GGGGTATTGC ACAATGGGCG
351 GAAGCCTGAT GCAGCGACGT CKCGTGAGGG ATGGAKGCCT TCKGGTTGTG
401 AACCTCTTTC GCCKGTGAAG AAGGTCCTGC TCCTTGTGGT GGGGYTGACG
451 GTAGCCGGGT TATGAA
```

Isolate **1** *2B* : *P. nigrescens*: 95.8% in 452 bp overlap (sequence length 452 bp)

See appendix 6.7.3 for sequence

Isolate **3** *1A* : *A. howellii*: 88.3% in 428 bp overlap (sequence length 429 bp)

```
  1 RSGSGYGYKC CTTGTKGGTG GGTTAGTGGC GAACGGGTGA GTATCACGTG
 51 AGTAACCTGC CCCCTGCTCC TGGATAACGC TCTGAAAGGG GTGCTAATAC
101 GGGGTGTGCT GGTGCTGCCG CATGGTGGTG CCGGGATAGG TTCCCTCTTT
151 GGGGGGTTCT GGTGGGGGAT GGGCTCGCGG CCTATCAGCT TGTTGGTGGG
201 GTGATGGCCT ACCAAGGCGG TGACGGGTAG CCGGCCTGAG AGGGTGGACG
251 GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTGCGGGA GGCAGCAGTG
301 GGGGGTATTG CGCAATGGGC GGAAGCCTGa CcCAGCGACG CCGCGTGAGG
351 GACGGAGGCc ttcgGGTTGT GAACCTCTTT CGCCGGTGGA GAAGGCGCCC
401 CCGTTTGGGG GTGCTGACTG AAGCCGGAT
```

Isolate **3** *2A* : *P. nigrescens*: 95.7% in 418 bp overlap (sequence length 418 bp)

See appendix 6.7.3 for sequence

Isolate **5** *1D* : *P. intermedia*: 97.5% in 439 bp overlap (sequence length 439 bp)

See appendix 6.7.3 for sequence

Isolate **6 1***A* : *S. sanguis*: 98.1% in 466 bp overlap (sequence length 466 bp)

```
  1 GGCGTGCCTA ATACATGCAA GTAGAACGCT GAAGAGAGGA GCTTGCTCTT
 51 CTTGGATGAG TTGCGAACGG GTGAGTAACG CGTAGGTAAC CTGCCTGGTA
```

```
101 GCGGGGGATA ACTATTGGAA ACGATAGCTA ATACCGCATA AAATTGATTA
151 TCGCATGATA ATTAATTGAA AGGTGCAAAT GCATCACTAC CAGATGGACC
201 TGCGTTGTAT TAGCTAGTTG GTGGGGTAAC GGCTCACCAA GGCGACGATA
251 CATAGCCGAC CTGAGAGGGT GATCGGCCAC ACTGGGACTG AGACACGGCC
301 CAGACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCGGCAA TGGACGGAAG
351 TCTGACCGAG CAACGCCGCG TGAGTGAAGA AGGTTTTCGG ATCGTAAAGC
401 TCTGTTGTAA GAGAAGAACG AGTGTGAGAG TGGAAAGTTC ACACTGTGAC
451 GGTATCTTAC CAGAAA
```

Isolate 7 *2B* : *S. gordonni*: 99.3 % in 417 bp overlap (sequence length 417 bp)

```
  1 CAGTTTATAC CGTAGCTTGC TACACCATAG ACTGTGAGTT GCGAACGGGT
 51 GAGTAACGCG TAGGTAACCT GCCTGGTAGC GGGGGATAAC TATTGGAAAC
101 GATAGCTAAT ACCGCATAAT ATTAATTATT GCATGATAAT TGATTGAAAG
151 ATGCAATTGC ATCACTACCA GATGGACCTG CGTTGTATTA GCTAGTAGGT
201 GAGGTAACGG CTCACCTAGG CGACGATACA TAGCCGACCT GAGAGGGTGA
251 TCGGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA
301 GTAGGGAATC TTCGGCAATG GACGAAAGTC TGACCGAGCA ACGCCGCGTG
351 AGTGAAGAAG GTTTTCGGAT CGTAAAGCTC TGTTGTAAGA GAAGAACGGG
401 TGTGAGAGTG GAAAGTT
```

Isolate **11** *1A* : *Peptostreptococcus* sp.: 98.0% in 361 bp overlap (sequence length 461 bp)

```
  1 GTGCCTAACA CATGCAAGTC GAGCGAGGGT TTGCTCAGTA TTGAGTATTC
 51 TAAGTCTAGA ATGTTCAATT CTGAGCAAAA CCAAGCGGCG GACGGGTGAG
101 TAACGCGTGG GTAACCTGCC CTATACACAT GGATAACATA CTGAAAAGTT
151 TACTAATACA TGATAATATA TATTTGCGGC ATCGCAGATA TATCAAAGTG
201 TTAGCGGTAT AGGATGGACC CGCGTCTGAT TAGCTAGTTG GTGAGATAAC
251 TGCCCACCAA GGCGACGATC AGTAGCCGAC CTGAGAGGGT GATCGGCCAC
301 ATTGGAACTG AGACACGGTC CAAACTCCTA CGGGAGGCAG CAGTGGGGAA
351 TATTGCACAA TGGGCGAAAG CCTGATGCAG CAACGCCGCG TGAACGATGA
401 AGGTCTTCGG ATCGTAAAGT TCTGTTGCAG GGGAAGATAA TGACGGTACC
451 CTGTGAGGAA G
```

Isolate **12** *2A* : *S. intermedius*: 93.6% in 435 bp overlap (sequence length 436 bp)

```
  1 GTACACCGTA GTTTACTACA CCGTACTTTG TGAGTCGCGA ACGGGTGAGT
 51 AACGCGTAGG TAACCTACCT TTTAGCGGGG GATAACTATT GGAAACGATA
101 GCTAATACCG CATAAGATAT CTTACTGCAT GGTAAGATAT TAAAAGATGC
151 AATTGCATCA CTAAGAGATG GACCTGCGTT GTATTAGCTA GTAGGTGAGG
201 TAACGGCTCA CCTAGGCGAC GATACATAGC CGACCTGAGA GGGTGATCGG
251 CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTAG
301 GGAATCTTCG GCAATGGGGG GAACCCTGAC CGAGCAACGC CGCGTGAGTG
351 AAGAAGGTTT TCGGATCGTA AAGCTCTGTT GTAAGAGAAG AACGAGTGTG
401 AGAGTGGAAA GTTCACACTG TGACGGTAAC TTACCA
```

Isolate **15** *1A* : *Bacillus* sp.: 100% in 466 bp overlap (sequence length 466 bp)

```
  1 GTGCCTAATA CATGcAAGTC GAGCGAATGG ATTAAGAGCT TGCTCTTATG
 51 AAGTTAGCGG CGGACGGGTG AGTAACACGT GGGTAACCTG CCCATAAGAC
101 TGGGATaACT CCGGGAAACC GGGGCTAATA CCGGATAACA TTTTGAACCG
151 CATGGTTCGA AATTGAAAGG CGGCTTCGGC TGTCACTTAT GGATGGACCC
201 GCGTCGCATT AGCTAGTTGG TGAGGTAACG GCTCACCAAG GCAACGATGC
251 GTAGCCGACC TGAGAGGGTG ATCGGCCACA CTGGGACTGA GACACGGCCC
301 AGACTCCTAC GGGAGGCAGC AGTAGGGAAT CTTCCGCAAT GGACGAAAGT
351 CTGACGGAGC AACGCCGCGT GAGTGATGAA GGCTTTCGGG TCGTAAAACT
401 CTGTTGTTAG GGAAGAACAA GTGCTAGTTG AATAAGCTGG CACCTTGACG
451 GTACCTAACC AGAAAG
```

6.22 CLINICAL *P. INTERMEDIA* ISOLATES - RESULTS OF RAPID ID 32 A AND RAPID ANA II SYSTEM

Table 6.22 Rapid ID 32 A results and Rapid ANA II system results for *Prevotella intermedia* isolated from PLS patients

ID 32 A				
TEST CODE	1 *2B* A	2 *3A* B	5 *1D* C	3 *2A* D
URE	-	-	-	-
ADH	-	-	-	-
αGAL	-	-	-	-
βGAL	-	-	-	-
βGP	-	-	-	-
αGLU	+	+	+	+
βGLU	-	-	-	-
αARA	-	-	-	-
βGUR	-	-	-	-
βNAG	-	-	-	-
MNE	+	-	-	+
RAF	+	+	-	+
GDC	-	-	-	-
FUC	-	+	+	+
NIT	-	-	-	-
IND	_	+	+	+
PAL	-	+	+	+
ArgA	+	+	-	+
ProA	-	-	-	-
LGA	+	+	+	+
PheA	-	-	-	-
LeuA	-	-	-	-
PyrA	-	-	-	-
TyrA	-	-	-	-
AlaA	+	+	+	+
GlyA	-	-	-	-
HisA	-	+	-	-
GGA	+	+	-	-
SerA	-	-	-	-
code	0406 6502 22	0404 6502 23	0400 6402 20	0400 6502 22
ID	*P. intermedia*			

ANA II				
TEST CODE	1 *2B* A	2 *3A* B	5 *1D* C	3 *2A* D
URE	-	-	-	-
BLTS	-	-	-	-
αARA	-	-	-	-
ONPG	-	-	-	-
αGLU	+	+	+	+
βGLU	-	-	-	-
αGAL	-	-	-	-
αFUC	+	+	+	+
NAG	-	-	-	-
PO4	+	+	+	+
LGY	+	+	+	+
GLY	-	-	-	-
PRO	-	-	-	-
PAL	-	-	-	-
ARG	-	+	+	+
SER	-	-	-	-
PYR	-	-	-	-
IND	+	+	+	+
code	022304	022344		
ID	*P. intermedia*			

KEY:
For test codes see tables 6.11 (Rapid ID 32 A) and 6.9 (RapID ANA II system).

6.23 PINLOS - RESULTS OF RAPID ID 32 A AND RAPID ANA II SYSTEM

Table 6.23 Rapid ID 32 A results for PINLOs in comparison with *Prevotella* sp.

TEST CODE	1 *2B* A	2 *3A* B	5 *1D* C	3 *2A* D	A391	HST 1156 HST 2160	*P. corporis* ATCC 33547 A350 A363	*P. pallens* NCTC 130Y2	*P. pallens* AHN 9423
URE	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-
αGAL	-	-	-	-	-	-	-	-	-
βGAL	-	-	-	-	-	-	-	-	-
βGP	-	-	-	-	-	-	-	-	
αGLU	+	+	+	+	+	+	+	+	+
βGLU	-	-	-	-	-	-	-	-	-
αARA	-	-	-	-	-	-	-	-	-
βGUR	-	-	-	-	-	-	-	-	-
βNAG	-	-	-	-	-	-	-	-	-
MNE	+	-	-	+	-	+	+	+	+
RAF	+	+	-	+	-	+	+	+	+
GDC	-	-	-	-	-	-	-	-	-
FUC	-	+	+	+	-	-	-	-	+
NIT	-	-	-	-	-	-	-	-	-
IND	_	+	+	+	+	+	-	+	+
PAL	-	+	+	+	-	-	+	+	+
ArgA	+	+	-	+	+	+	+	+	+
ProA	-	-	-	-	-	-	-	-	-
LGA	+	+	+	+	+	+	+	+	+
PheA	-	-	-	-	-	-	-	-	-
LeuA	-	-	-	-	-	-	-	-	-
PyrA	-	-	-	-	-	-	-	-	-
TyrA	-	-	-	-	-	-	-	-	-
AlaA	+	+	+	+	+	+	+	+	+
GlyA	-	-	-	-	-	-	-	-	-
HisA	-	+	-	-	+	-	-	-	-
GGA	+	+	-	-	+	+	+	+	+
SerA	-	-	-	-	-	-	-	-	-
Micro-code	0406 6502 22	0404 6502 23	0400 6402 20	0406 6502 22	0400 6502 23	0406 6502 02	0406 4502 02	0406 6502 02	0406 6502 22
ID	*P. intermedia*								

KEY: For test codes see table 6.11.

Table 6.24 Rapid ANA II system results for PINLOs in comparison with *Prevotella* sp.

TEST CODE	1 *2B* A	2 *3A* B	5 *1D* C	3 *2A* D	A391 HST 1156 HST 2160	*P. corporis* ATCC 33547 A350 A363	*P. pallens* NCTC 130Y2	*P. pallens* AHN 9423
URE	-	-	-	-	-	-	-	-
BLTS	-	-	-	-	-	-	-	-
αARA	-	-	-	-	-	-	-	-
ONPG	-	-	-	-	-	-	-	-
αGLU	+	+	+	+	+	+	+	+
βGLU	-	-	-	-	-	-	-	-
αGAL	-	-	-	-	-	-	-	-
αFUC	+	+	+	+	-	-	-	+
NAG	-	-	-	-	-	-	-	-
PO4	+	+	+	+	+	+	+	+
LGY	+	+	+	+	+	+	+	+
GLY	-	-	-	-	-	-	-	-
PRO	-	-	-	-	-	-	-	-
PAL	-	-	-	-	-	-	-	-
ARG	-	+	+	+	+	+	+	+
SER	-	-	-	-	-	-	+	-
PYR	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	-	+	+
Code	022304	022344			020344	020340	020345	022344
ID	*P. intermedia*					*P. corporis*	*P. intermedia*	

KEY: For test codes see table 6.9.

6.24 ELISA ASSAY USING PAPILLON-LEFÈVRE SYNDROME PLAQUE SAMPLES

Periodontal pocket samples were resuspended in PBS containing 0.01% (w/v) thiomersal, frozen and sent to Dr. S. Hamlet at the Division of Oral Biology, University of Queensland, Brisbane, Australia. An ELISA assay (enzyme-linked immunosorbent assay) was used to measure the levels of the periodontopathogens *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *P. intermedia* in the plaque samples.

6.24.1 METHOD

Plaque samples were dispersed by vortexing and sonication for 5 seconds. The suspension was then diluted in an equal volume of 0.1 M carbonate buffer pH 9.6 and 100 µl volumes used to fill the wells in the microtiter plate. A standard curve of either *A. actinomycetemcomitans* Y4 or *P. gingivalis* FDC-381 or *P. intermedia* ATCC 25611 was also transferred to the plate. The standard curve contained a range of 150 - $9x10^4$ cells/ml. Microtiter plates were incubated overnight at 4°C and washed three times with PBS (0.05%) - Tween 20 (PBS-T). Plates were incubated for 1 h at room temperature with PBS-T containing 1% foetal calf serum and washed three times in PBS-T. A specific antibody to one species was purified and conjugated to horse radish peroxidase. Diluted antibody was added to the wells and the plates were incubated at room temperature for 2 h before washing three times with PBS-T. To facilitate a colour change, 150 µl of 2.5 mM o-tolidine in 100 mM phosphate citrate buffer pH 4.5 containing 0.025 mM EDTA activated by 3% H_2O_2 was added. After 10 min the reaction was stopped by adding 50 µl of 1M HCl. The plates were read in a microplate reader at 450 nm and 655 nm.

6.24.2 RESULTS

The findings are summarised here. Each species was either absent or present in low levels.

- *P. gingivalis* occurred in every sample from patient 1 but was absent from all samples from patient 2.
- *Actinobacillus actinomycetemcomitans* occurred in only one sample which was from patient 2.
- *P. intermedia* was found in 1/6 samples from patient 2 and 3/7 from patient 1.